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(74) Agents: **CORUZZI, Laura, A.** et al.; Pennie & Edmonds
LLP, 1155 Avenue of the Americas, New York, NY 10036
(US).

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(71) Applicant (for all designated States except US): **THE RE-
SEARCH FOUNDATION OF THE STATE UNIVER-
SITY OF NEW YORK** [US/US]; P.O. Box 9, Albany, NY
12201-0009 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **COHEN, Stanley**,
N. [US/US]; Department of Genetics School of Medicine,
Stanford University, Stanford, CA 94305-5120 (US).
CARTER, Carol [US/US]; 33 Renwick Avenue, Hunt-
ington, NY 11743 (US). **GOFF, Arthur** [US/US]; 279
Radio Avenue, Miller Place, NY 11764 (US). **EHRlich,**
Lorna [US/US]; #1 Colgate Court, Shoreham, NY 11786
(US).

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(54) Title: TSG101 AS INHIBITOR OF HIV PRODUCTION

(57) Abstract: The present invention provides methods for identifying peptides in a mammalian Tsg101 protein that binds to the PTAPP motif or L domain of human immunodeficiency virus type I (HIV-1). Such peptides can be used to inhibit Tsg101-HIV Gag binding, and is therefore effective in reducing HIV particle production. The invention also provides the peptides identified by the method of the invention and to method of using such peptides for treating HIV infection.

TSG101 AS INHIBITOR OF HIV PRODUCTION

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This application claims the benefit, under 35 U.S.C. §119(e), of U.S. Provisional Patent Application No. 60/292,761, filed on May 21, 2001, which is incorporated herein by reference in its entirety.

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This invention was made with U.S. Government support. Accordingly, the U.S. Government has certain rights in the invention.

1. FIELD OF THE INVENTION

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The present invention relates to methods for identifying peptides in a mammalian Tsg101 protein, which is effective in reducing retroviral particle production, e.g., HIV particle production. The invention also relates to the peptides identified by the method of the invention and to method of using such peptides for treating retroviral infection, HIV infection.

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2. BACKGROUND OF THE INVENTION

The Pr55^{Gag} protein of the human immunodeficiency virus type I (HIV-1) contains all of the information required for transport to assembly sites on the plasma membrane, association with genomic RNA, and release into extracellular space (see, e.g., Swannstrom, R. & Wills, J. W., 1997, in *Retroviruses*, eds. Coffin, J. M., Hughes, S. H. & Varmus, H. E., Cold Spring Harbor Laboratory Press, New York, pp. 263-334). However, although Gag is sufficient for viral assembly, cellular proteins are likely to facilitate the process. Several cellular proteins can be recovered from purified virions, suggesting proximity to the assembling particle (see, e.g., Arthur, L. O., Bess, J. W., Jr., Sowder, R. C. I., Benveniste, R. E., Mann, D. L., Chermann, J.-C. & Henderson, L. E., 1992, *Science* 258, 1935-1938; and Ott, D. E., Coren, L. V., Kane, B. P., Busch, L. K., Johnson, D. J. Sowder, R. C. I., Chertova, E. N., Arthur, L. O. & Henderson, L. E., 1996, *J. Virol.* 70, 7734-7743). Others interact directly with Pr55^{Gag} (see, e.g., Luban, B., Bossolt, K. L., Franke, E. K., Kalpana, G. V. & Goff, S. P., 1993, *Cell* 73, 1067-1078). Interaction with still others is implied, since Gag contains post-translational modifications (see, e.g., Bryant, M. & Ratner, L., 1990, *Proc. Natl. Acad. Sci. USA* 87, 523-527; Camaur, D., Gallay, P., Swinger, S. & Trono, D., 1997, *J. Virol.* 71, 6834-6841; Göttinger, H. G., Sodroski, J. G. & Haseltine, W. A., 1989,

- Proc. Natl. Acad. Sci. USA* 86,5781-5785; Ott, D. E., Coren, L. V., Copeland, T. D., Kane, B. P., Johnson, D. G., Sowder, R. C., 2nd, Yoshinaka, Y., Oroszlan, S., Arthur, L. O. & Henderson, L. E., 1998, *J. Virol.* 72, 2962-2968). There are now reports that the region in Gag required for release of mature particles, the late (L) domain (see, e.g., Wills, J. W.,
- 5 Cameron, C. E., Wilson, C. B., Xiang, Y., Bennett, R. P. & Leis, J., 1994, *J. Virol.* 68, 6605-6618; Göttinger, H. G., Dorfman, T., Sodroski, J. G. & Haseltine, W. A., 1991, *Proc. Natl. Acad. Sci. USA* 88, 3195-3199; Huang, M., Orenstein, J. M., Martin, M. A. & Freed, E. O., 1995, *J. Virol.* 69, 6810-6818) directs the interaction of the protein with the ubiquitination machinery (see, e.g., Schubert, U., Ott, D. E., Chertova, E. N., Welker, R.,
- 10 Tessmer, U., Princiotta, M. F., Bennink, J. R., Krausslich, H. G. & Yewdell, J. W., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13057-13062; Strack, B., Calistri, A., Accola, M. A., Palu, G. & Gottlinger, H. G., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13063-13068; Vogt, V. M., 2000, *Proc. Natl. Acad. Sci. USA* 97, 12945-12947; Ott, D. E., Coren, L. V., Chertova, E. N., Gagliardi, T. D. & Schubert, U., 2000, *Virology* 278, 111-121, Patnaik, A., Chau, V. &
- 15 Wills, J. W., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13069-13074). Based on its sequence and recent studies, Tsg101 is an ubiquitin (Ub)-conjugating E2 enzyme variant (UEV) protein involved in regulation of intracellular trafficking, transcriptional regulation, and cell cycle control (see, e.g., Babst, M., Odorizzi, G., Estepa, E. J. & Emr, S. D., 2000, *Traffic* 1,242-258; Lemmon, S. K. & Traub, L. M., 2000, *Curr. Opin Cell Biol.* 12,457-466, Xie,
- 20 W., Li, L. & Cohen, S. N., 1998, *Proc. Natl. Acad. Sci. USA* 95, 1595-1600; Zhong, Q., Chen, Y., Jones, D. & Lee, W. H., 1998, *Cancer Res.* 58,2699-2702; Sun, Z., Pan, S., Hope, W. X., Cohen, S. N. & Balk, S. P., 1999, *Cancer* 86,689- 96). UEV proteins lack the critical Cys residue essential for conjugation and transfer of Ub to protein substrates or Ub-ligating (E3) enzymes (Koonin, E. V. & Abagyan, R. A., 1997, *Nat. Genet.* 16,330-331;
- 25 Ponting, C. P., Cai, Y.-D. & Bork, P., 1997, *J. Mol. Med.* 75,467-469). They are highly conserved in evolution and constitute a novel family of proteins structurally related to, but distinct from, E2 enzymes.

All retroviruses have in common 3 genes, *gag*, *pol*, and *env*, which specify the structural and enzymatic functions of the virus (see, e.g., Swanstrom, R. & Wills, J. W.,

30 1997, in *Retroviruses*, eds. Coffin, J. M., Hughes, S. H. & Varmus, H. E., Cold Spring Harbor Laboratory Press, New York, pp. 263-334). The *gag* gene alone is sufficient for assembly and release of immature virus-like particles from infected cells. Maturation to form the infectious particle requires a viral-encoded protease (PR) encoded in *pol*. The *gag*-encoded protein (Gag) contains distinct domains involved in assembly and release. There is

35 a plasma membrane-binding (M) domain located in the N-terminal matrix (MA) region, a

capsid (CA) domain that forms a genome-encasing core structure, a protein interaction (I) domain in the nucleocapsid (NC) region, and a late (L) domain, required for release by budding from the plasma membrane. The L domain is a Pro-rich motif that is highly conserved in retroviruses; other enveloped viruses, including rhabdo-, fib-, and Epstein Barr viruses, and cellular proteins also have Pro-rich motifs (Harty, R. N., Brown, M. E., Wang, G., Huibregtse, I. & Hayes, F. P., 2000, *Proc. Natl. Acad. Sci. USA* **97**, 13871-13876; Wills, J. W., Cameron, C. E., Wilson, C. B., Xiang, Y., Bennett, R. P. & Leis, J., 1994, *J. Virol.* **68**, 6605-6618; Göttinger, H. G., Dorfman, T., Sodroski, J. G. & Haseltine, W. A., 1991, *Proc. Natl. Acad. Sci. USA* **88**, 3195-3199; Huang, M., Orenstein, J. M., Martin, M. A. & Freed, E. O., 1995, *J. Virol.* **69**, 6810-6818; Ikeda, M., Ikeda, A., Longan, L.C. & Longnecker, R., 2000, *Virol.* **268**, 178-191; Yasuda, J. & Hunter, E., 1998, *J. Virol.* **72**, 4095-4103; Harty, R.N., Paragas, J., Sudol, M. & Palese, P., 1999, *J. Virol.* **73**, 2921-2929). The L domains in retroviruses differ in amino acid sequence and location within the respective viral structural proteins, but are functionally exchangeable (Wills, J. W., Cameron, C. E., Wilson, C. B., Xiang, Y., Bennett, R. P. & Leis, J., 1994, *J. Virol.* **68**, 6605-6618; Parent, L.J., Bennett, R.P., Craven, R.C., Nelle, T.D., Krishna, N.K., Bowzard, J.B., Wilson, C.B., Puffer, B.A., Montelaro, R.C. & Wills, J.W., 1995, *J. Virol.* **69**, 5455-5460), suggesting commonality of function.

The protein product of the TSG101 gene was originally identified by the reversible neoplasia associated with its functional inactivation in murine fibroblasts (Li, L. & Cohen, S. N., 1996, *Cell* **85**, 319-329). Sequence analysis has suggested (Li, L. & Cohen, S. N., 1996, *Cell* **85**, 319-329; Koonin, E. V. & Abagyan, R. A., 1997, *Nat. Genet.* **16**, 330-331; Ponting, C. P., Cai, Y.-D. & Bork, P., 1997, *J. Mol. Med.* **75**, 467-469), and experimental evidence has shown, that Tsg101 can function in both the modulation of transcription (Sun, Z., Pan, J., Hope, W. X., Cohen, S. N. & Balk, S. P., 1999, *Cancer* **86**, 689-96; Watanabe, M., Yanagi, Y., Masahiro, Y., Yano, T., Yoshikawa, H., Yanagisawa, J., & Kato, S., 1998, *Biochem. Biophys. Res. Commun.* **245**, 900-905; Hittleman, A.B., Burakov, D., Iniguez-Lluhi, J.A., Freedman, L.P., & Garabedian, M.J., 1999, *EMBO J.* **18**, 5380-5388) and the inhibition of ubiquitination and protein decay (Li, L., Liao, J., Ruland, J., Mak, T.W., & Cohen, S.N., 2001, *Proc. Natl. Acad. Sci. USA* **98**, 1619-1624). The latter effects are mediated by an N-terminal region that contains a ubiquitin (Ub) conjugase (E2)-like domain, but lacks an active site Cys residue crucial to Ub conjugation. The structure of the UEV domain of Tsg101 and its modes of interaction with Ub and PTAP have also been reported (Pornillos et al., 2002, *EMBO J.* **21**:2397-2406).

Cells deficient in Tsg101 show a variety of nuclear, microtubule, and mitotic spindle abnormalities (Xie, W., Li, L. & Cohen, S. N., 1998, *Proc. Natl. Acad. Sci. USA* **95**, 1595-1600; Zhong, Q., Chen, Y., Jones, D. & Lee, W. H., 1998, *Cancer Res.* **58**, 2699-2702), and *tsg101* null mutant mice show defective cell proliferation and early embryonic death

5 (Ruland, J., Sirard, C., Elia, A., MacPherson, D., Wakeham, A., Li, L., de la Pompa, J.L., Cohen, S.N., & Mak, T.W., 2001, *Proc. Natl. Acad. Sci. USA* **98**, 1859-1864). The steady state level of Tsg101 normally is controlled post-translationally within a narrow range in cultured murine and human cell lines (Feng, G.H., Lih, C.-J., & Cohen, S.N., 2000, *Cancer Research* **60**, 1736-1741) and over-expression from an adventitious promoter, as well as

10 *tsg101* deficiency, can lead to neoplastic transformation (Li, L. & Cohen, S. N., 1996, *Cell* **85**, 319-329). Regulation of the Tsg101 protein level, which can be affected by the Ub ligase, Mdm2 (Li, L., Liao, J., Ruland, J., Mak, T.W., & Cohen, S.N., 2001, *Proc. Natl. Acad. Sci. USA* **98**, 1619-1624), requires a C-terminal Tsg101 sequence that is evolutionarily highly conserved in organisms as disparate as humans, *C. elegans*, *S. pombe*,

15 and *D. melanogaster* (Feng, G.H., Lih, C.-J., & Cohen, S.N., 2000, *Cancer Research* **60**, 1736-1741; Bishop N and Woodman P., 2001, *J Biol Chem.* **276**:11735-42).

Stp22p/Vps23, a class E vacuolar protein sorting (*vps*) protein in *S. cerevisiae*, has been identified as a Tsg101 orthologue and Tsg 101 itself has been implicated in the trafficking of membrane-associated proteins (Bishop N and Woodman P., 2001, *J Biol*

20 *Chem.* **276**:11735-42; Babst, M., Odorizzi, G., Estepa, E. J. & Emr, S. D., 2000, *Traffic* **1**, 242-258; Li, Y., Kane, T., Tipper, C., Spatrick, P. & Jenness, D.D., 1999, *Mol. & Cell. Biol.* **19**, 3588-3599). It has also been shown that SL6 cells are defective in sorting of multiple surface-bound proteins (Babst, M., Odorizzi, G., Estepa, E. J. & Emr, S. D., 2000, *Traffic* **1**, 242-258).

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Citation of references hereinabove shall not be construed as an admission that such references are prior art to the present invention.

3. SUMMARY OF THE INVENTION

30 The present invention provides methods for identifying peptides in a mammalian Tsg101 protein that bind to the PTAP motif or L domain of human immunodeficiency virus type I (HIV-1). Such peptides can be used to inhibit Tsg101-HIV Gag binding, and are therefore effective in reducing HIV particle production. In a preferred embodiment of the invention, peptides in a mammalian *tsg101* protein are identified by a method comprising

35 (a) measuring a level of HIV viral particles released in a culture of mammalian cells,

wherein said mammalian cells comprise an expression construct comprising a portion of the coding sequence of a mammalian *tsg101* gene such that said mammalian cells express a gene product encoded by said portion of said coding sequence of said mammalian *tsg101* gene and are infected by HIV virus; and (b) comparing the level of HIV viral particles to a level of HIV viral particles released in a culture of control mammalian cells which do not comprise an expression construct comprising a portion of a coding sequence for a mammalian *tsg101* gene under similar conditions. In the method, the level of HIV viral particles measured in step (a) compared to the level of HIV viral particles in the culture of control mammalian cells below a predetermined threshold level identify the gene product encoded by the portion of the coding sequence of the mammalian *tsg101* gene as effective in reducing HIV particle production. In a preferred embodiment, the level of HIV viral particles released in a culture of mammalian cells and the level of HIV viral particles released in a culture of control mammalian cells are represented by measured levels of particle associated p24. In preferred embodiments of the invention, the predetermined threshold level is a two-fold, four-fold, 90%, 95%, 99%, or 99.5% reduction of the level of HIV viral particles measured in step (a) compared to the level of HIV viral particles of the culture of control mammalian cells.

In another preferred embodiment of the invention, peptides in a mammalian *tsg101* protein are identified by a method comprising (a) measuring a level of HIV viral particle released in a culture of mammalian cells, wherein the mammalian cells comprise (i) an expression construct comprising a portion of the coding sequence of a mammalian *tsg101* gene and (ii) an expression construct comprising the HIV *gag* coding sequence such that the mammalian cells express a gene product encoded by the portion of the coding sequence of the mammalian *tsg101* gene and a gene product encoded by the HIV *gag* gene; and (b) comparing the level of HIV viral particle released to a level of HIV viral particle released in a culture of control mammalian cells under similar conditions, wherein the control mammalian cells comprise one or more expression constructs comprising the HIV *gag* coding sequence such that the mammalian cells express a gene product encoded by the HIV *gag* gene, and wherein the control mammalian cells do not comprise an expression construct comprising a portion of a coding sequence for a mammalian *tsg101* gene. In still another preferred embodiment of the invention, peptides in a mammalian *tsg101* protein are identified by a method comprising (a) measuring a level of HIV viral particles released in a culture of mammalian cells, wherein the mammalian cells comprise (i) an expression construct comprising a portion of the coding sequence of a mammalian *tsg101* gene and (ii) one or more expression constructs comprising the HIV *gag*, *pol*, and *rev* coding sequences

such that the mammalian cells express a gene product encoded by the portion of the coding sequence of the mammalian *tsg101* gene and gene products encoded by the HIV *gag*, *pol* and *rev* genes; and (b) comparing the level of HIV viral particles released to a level of HIV viral particles released in a culture of control mammalian cells under similar conditions,

5 wherein the control mammalian cells comprise one or more expression constructs comprising the HIV *gag*, *pol*, and *rev* coding sequences such that the mammalian cells express gene products encoded by the HIV *gag*, *pol* and *rev* genes, and wherein the control mammalian cells do not comprise an expression construct comprising a portion of a coding sequence for a mammalian *tsg101* gene. In these embodiments, the level of HIV viral

10 particles measured in step (a) compared to the level of HIV viral particles released in the culture of control mammalian cells below a predetermined threshold level identify the gene product encoded by the portion of the coding sequence of the mammalian *tsg101* gene as effective in reducing HIV particle production. In a preferred embodiment, the level of HIV viral particles released in a culture of mammalian cells and the level of HIV viral particles

15 released in a culture of control mammalian cells are represented by measured levels of particle associated p24. In preferred embodiments of the invention, the predetermined threshold level is a two-fold, four-fold, 90%, 95%, 99%, or 99.5% reduction of the level of particle associated p24 measured in step (a) compared to the level of particle associated p24 of the culture of control mammalian cells.

20 The invention also provides a method for identifying a peptide or fragment derived from a mammalian Tsg101 protein, which is effective in reducing production of retroviral particles, e.g., particles of a lentivirus, including but not limited to HIV-1 and HIV-2, Simian Immunodeficiency Virus (SIV). The method comprises identifying a peptide that binds to a peptide comprising the PTAP motif of a retroviral protein, e.g., a HIV Gag

25 protein. In a preferred embodiment, the peptide comprising the PTAP motif is the peptide of SEQ ID NO:4. In another preferred embodiment, the peptide comprising the PTAP motif is a HIV Gag protein.

The invention also provides a method for identifying a peptide comprising a PTAP motif, which is effective in reducing production of retroviral particles, e.g., particles of a

30 lentivirus, including but not limited to HIV-1 and HIV-2, Simian Immunodeficiency Virus (SIV). The method comprises identifying a peptide identifying a peptide comprising a PTAP motif which binds to a TSG101 protein. In a preferred embodiment, the peptide comprises peptide comprises the sequence of SEQ ID NO:4.

The invention also provides the peptides identified by the method of the invention

35 and to method of using such peptides for treating HIV infection.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG 1. Identification of the region in PR55^{Gag} required for Tsg101 binding using the 2-hybrid assay. Reporter gene activation was quantified by determination of β -galactosidase units. FIG.1A, The interaction of Gag and pl-p6 with Tsg101.

5 β -Galactosidase activity of Gag and pl-p6 were equivalent in two independent trials. FIG. 1B, The interaction of pl-p6 with Tsg101 (taken as 100%) ranged from ~10 to 30 β -galactosidase units in twenty independent trials. Negative interactions were equivalent to that obtained when pl-p6 was co-transformed with vector lacking Tsg101 (<0.3 units). The figure shows averaged values obtained for mutants in six independent trials as a percentage

10 of the wild-type interaction +/- 1%.

FIG. 2. Binding of PR55^{Gag} and Tsg101 *in vitro*. FIG. 2A, Autoradiography to detect immune-captured radiolabeled Tsg101. Lane 1, Tsg101 synthesized in RRL. Lanes 2 to 9, Determination of binding of radioactive Tsg101 to unlabeled Pr55GS bound to anti-T7 (lane

15 3), anti-CA (lanes 7, 8), or anti-p6 (lane 9) IgG immobilized on protein A beads. Antibodies are as defined in Materials and Methods. The amount of RRL used in lanes 2 to 9 was 5-fold greater than the amount used in lane 1. FIG. 2B, Confirmation of the presence of PR55^{Gag} on the beads by Western analysis. A monoclonal antibody against an antigenic site in the CA domain was used to visualize the Gag proteins immunoprecipitated with the

20 antibodies used in A. Molecular weight markers (kDa) are on the left of the panels.

FIG. 3. Co-immune precipitation of PR55^{Gag} and Tsg101 from cytoplasmic extracts. FIG. 3A, Total cytoplasmic extract. Extracts Were prepared from cells transfected with rev (lane 1), *rev*, *gag* and *pol* (lane 2), or *rev*, *gag* Δ *p6* and *pol* (lane 3). FIG. 3B,

25 Immunoprecipitation with anti-Tsg101 monoclonal antibody using extracts of cells transfected with *rev* (lane 1); *rev*, wild-type *gag* and *pol* (lane 2); or *rev*, *gag* Δ *p6* and *pol* (lane 3) as detected by the anti-NC polyclonal antibody. The blot was re-probed with an anti-Tsgl 01 polyclonal antibody to confirm the presence of Tsg101 in the immune-precipitates (lanes 4-6). FIG. 3C, Immunoprecipitation with anti-CA polyclonal

30 antibody using extracts from cells transfected with *rev* (lane 1) or *rev*, *gag* and *pol* (lane 2) as detected by anti-Tsgl01 monoclonal antibody. The blot was re-probed with anti-CA monoclonal antibody to confirm the presence of Gag in the immune-precipitate (lanes 3, 4). Molecular weight markers (kDa) are on the left of the panels.

35 FIG. 4. Identification of the region in Tsg101 required for Pr55^{Gag} binding.

Truncation (FIG. 4A) and substitution (FIGS. 4B and 4C) mutants of Tsg101 were tested in the 2-hybrid assay for interaction with the pl-p6 fusion protein. The figure shows averaged values as a percentage of the wild-type interaction with +/- 2-6% error. Notations are as in legend to Fig. 1.

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FIG. 5. Effects of Tsg101 deficiency on virus production and cellular localization. FIG. 5A, NIH 3T3 and SL6 lysates prepared from *gag*-transfected cells were separated by SDS-PAGE, stained by Coomassie blue for total protein (lanes 1 & 2), and analyzed by Western blotting for Na⁺K⁺ATPase, actin, and Tsg101 (lanes 3 & 4). FIGS. 5B-5D, Western blots of (5B) viral particles; (5C) S1 extracts of NIH 3T3 and SL6 cells; *Inset*, S100 (lane 1) and P100 (lane 2) fractions of pHIV-transfected NIH 3T3 cells; (5D) S1 and P1 extracts of SL6 cells. The blots were probed with anti-CA rabbit serum (FIGS. 5B and 5C) or anti- NC goat antiserum. (FIG. 5D). Equivalent samples for Western analysis were determined by Biorad assay.

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FIG. 6. Effect of wild-type Tsg101 over expression on release of HIV-1 particles. FIG. 6A, Induced expression of wild-type Tsg101_{FLAG}. S1 fractions prepared from transfected COS-1 cells were analyzed by Western blotting with anti-Tsg101 antibody. *pa*, ponasterone inducer. FIG. 6B, Effect of Tsg101-FLAG over expression on HIV-1 release from COS-1 cells, analyzed by Western blotting of particles with anti-CA antibody. Migration positions of molecular size markers are indicated on the left.

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FIG. 7. Effect of mutated Tsg101 over expression on release of HIV-1 particles. FIG. 7A, Induced expression of Tsg101_{FLAGΔ348-351G}. FIG. 7B, Effect of Tsg101-FLAG over expression on HIV-1 particle production. S1 fractions and viral particles were analyzed as described in the legend to FIG. 6.

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FIG. 8. Immunoprecipitation of viral proteins in cytoplasmic extracts with anti-Tsg101 antibody. FIG. 8A, Cytoplasmic extracts, prepared from mock- (lanes 1 & 2) and *gag*- (lanes 3 & 4) transfected COS-1 cells expressing FLAG-tagged wild-type (lanes 1 & 3) or mutated (lanes 2 & 4) Tsg101, were analyzed by Western blotting with anti-p6 antibody. Expression was induced using 2 μg DNA encoding *tsg101* and 5 μM *pa*. FIGS. 8B-8D, The extracts were immunoprecipitated with anti-Tsg101 rabbit polyclonal antibody and probed for proteins with (8B) anti-p6 antibody; (8C) anti-FLAG antibody; and (8D) antiTsg101 mouse monoclonal antibody.

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FIG. 9. Effect of Tsg101 over expression on intracellular steady-state level of viral proteins. Cytoplasmic extracts were prepared from cells expressing Rev- (FIG. 9A, lanes 1 & 3), wild-type Gag- (FIGS. 9B & 9C, lanes 1 & 3), or Gag Δ p6- (FIGS. 9B & 9C, lanes 2 & 4) and FLAG-tagged wild-type (FIG. 9B) or mutated (FIG. 9C) Tsg101. S1 (lanes 1 & 2) and P1 (lanes 3 & 4) fractions were analyzed by Western blotting with anti-CA antibody.

5. DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, it has been discovered that the PTAP motif or L domain of human immunodeficiency virus type I (HIV-1), which is located in the C-terminal p6 region of the Gag precursor polypeptide, binds to the protein product of the *tsg101* gene *in vitro* and in the cytoplasm of transfected cells. The present invention provides an assay for testing molecules, e.g., peptides comprising at least four contiguous amino acids of the PTAP motif, and which molecules inhibit Tsg101-HIV Gag binding. Preferably, the assay of the invention is used to identify molecules, e.g., peptides, which can achieve a two-fold, four-fold, more than 90%, more than 95%, more than 99%, or more than 99.5% reduction of HIV viral release. Molecules, e.g., peptides, identified by the assay may be used as therapeutic compositions for treating patients infected with HIV-1 or other retrovirus.

It has also been surprisingly found that perturbation of the steady-state level of Tsg101 protein by under- or over-expression of the Tsg101 protein negatively affects release of HIV-1 virus-like particles from murine and primate cells. The present invention therefore provides a pharmaceutical composition comprising one or more pharmaceutically acceptable carriers or diluents and a mammalian Tsg101 protein. In another aspect of the invention, assays are provided for identifying fragments of a mammalian Tsg101 protein which are effective in reducing HIV particle production. As used herein, "HIV particle" encompasses both mature and immature virus-like particles. A fragment of Tsg101, herein referred to as a "peptide" of Tsg101, identified by a subject assay as effective in reducing HIV particle production, is useful as a therapeutic composition for treating a patient infected with HIV-1 or other retrovirus. Pharmaceutical compositions comprising one or more subject peptides and one or more pharmaceutically acceptable carriers or diluents are therefore also provided.

Thus, in one embodiment of the present invention, there is provided a method for identifying a peptide derived from a mammalian Tsg101 protein, which peptide is effective in reducing HIV particle production. The method comprises the steps of: (a) introducing into a mammalian cell or cells, a first expression construct wherein the first expression

construct comprises a portion of the coding sequence for a mammalian *tsg101* gene; (b) introducing into the same mammalian cell or cells either HIV virus or one or more expression vectors comprising the HIV *gag*, *pol* and *rev* coding sequences; (c) incubating the transfected mammalian cell or cells in a suitable media for a sufficient time and under conditions sufficient to obtain a mammalian cell culture wherein the mammalian cells express a gene product encoded by the portion of a coding sequence for a mammalian *tsg101* gene, and gene products encoded by the HIV *gag*, *pol*, and *rev* genes. In the case where one or more expression vectors comprising the HIV *gag*, *pol*, and *rev* genes, are used to transfect a mammalian cell or cells, the level of particle associated p24 (capsid protein) may be measured. A finding of a reduced level of particle associated p24, when compared to a control mammalian cell culture which has not been transfected with the expression construct comprising a portion of the coding sequence for a mammalian *tsg101* gene, correlates with the identification of a Tsg101 peptide effective in reducing HIV particle formation. In the case where HIV virus is added to the mammalian cell or cells, particle associated p24 may be measured and a correlation made, as described above. Alternatively, when HIV virus is added to the mammalian cell or cells, HIV particles released from the cells may be quantified. A finding of a reduction in HIV particle production, when compared to a control mammalian cell culture which has not been transfected with the expression construct comprising a portion of the coding sequence for a mammalian *tsg101* gene, correlates with the identification of a peptide effective in reducing HIV particle production.

In another embodiment of the invention, peptides in a mammalian tsg101 protein are identified by a method comprising (a) measuring a level of HIV viral particles released in a culture of mammalian cells, wherein said mammalian cells comprise an expression construct comprising a portion of the coding sequence of a mammalian *tsg101* gene such that said mammalian cells express a gene product encoded by said portion of said coding sequence of said mammalian *tsg101* gene and are infected by HIV virus; and (b) comparing the level of HIV particles to a level of HIV particles released in a culture of control mammalian cells which do not comprise an expression construct comprising a portion of a coding sequence for a mammalian *tsg101* gene under similar conditions. In the method, the level of HIV particles measured in step (a) compared to the level of HIV particles of the culture of control mammalian cells below a predetermined threshold level identify the gene product encoded by the portion of the coding sequence of the mammalian *tsg101* gene as effective in reducing HIV particle production.

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In still another embodiment of the invention, peptides in a mammalian *tsg101* protein are identified by a method comprising (a) measuring a level of HIV viral particle released in a culture of mammalian cells, wherein the mammalian cells comprise (i) an expression construct comprising a portion of the coding sequence of a mammalian *tsg101* gene and (ii) an expression construct comprising the HIV *gag* coding sequence such that the mammalian cells express a gene product encoded by the portion of the coding sequence of the mammalian *tsg101* gene and a gene product encoded by the HIV *gag* gene; and (b) comparing the level of HIV viral particle released to a level of HIV viral particle released in a culture of control mammalian cells under similar conditions, wherein the control mammalian cells comprise one or more expression constructs comprising the HIV *gag* coding sequence such that the mammalian cells express a gene product encoded by the HIV *gag* gene, and wherein the control mammalian cells do not comprise an expression construct comprising a portion of a coding sequence for a mammalian *tsg101* gene.

In still another embodiment of the invention, peptides in a mammalian *tsg101* protein are identified by a method comprising (a) measuring a level of HIV particles released in a culture of mammalian cells, wherein the mammalian cells comprise (i) an expression construct comprising a portion of the coding sequence of a mammalian *tsg101* gene and (ii) one or more expression constructs comprising the HIV *gag*, *pol*, and *rev* coding sequences such that the mammalian cells express a gene product encoded by the portion of the coding sequence of the mammalian *tsg101* gene and gene products encoded by the HIV *gag*, *pol* and *rev* genes; and (b) comparing the level of HIV particles released to a level of HIV particles released in a culture of control mammalian cells under similar conditions, wherein the control mammalian cells comprise one or more expression constructs comprising the HIV *gag*, *pol*, and *rev* coding sequences such that the mammalian cells express gene products encoded by the HIV *gag*, *pol* and *rev* genes, and wherein the control mammalian cells do not comprise an expression construct comprising a portion of a coding sequence for a mammalian *tsg101* gene. In these embodiments, the level of HIV particles measured in step (a) compared to the level of HIV particles released in the culture of control mammalian cells below a predetermined threshold level identify the gene product encoded by the portion of the coding sequence of the mammalian *tsg101* gene as effective in reducing HIV particle production.

In preferred embodiments of the invention, the peptide identified causes at least a two-fold, four-fold, 90%, 95%, 99%, or 99.5% reduction of the level of HIV particles released or reduction of the level of particle associated p24 in the culture.

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In an alternative embodiment, a method for identifying a peptide derived from a mammalian Tsg101 protein, which peptide is effective in reducing HIV particle production may be performed as described above except that instead of using an expression construct comprising a portion of a coding sequence for a mammalian *tsg101* gene, random fragments
5 (peptides) of a mammalian Tsg101 protein are generated and added to a mammalian cell or cells which cells are also infected with HIV or transfected with one or more expression constructs comprising the HIV *gag*, *pol*, and *rev* coding sequences. Tsg101 fragments (peptides) may be introduced into mammalian cells using well known methods of permeation. It will be apparent to a person of ordinary skill in the art that this embodiment
10 is also applicable for identifying other molecules that is effective in reducing HIV or other viral particle production. Therefore, in another aspect of the invention, a method for identifying molecules that is effective in reducing HIV or other viral particle production is provided.

In another embodiment, the invention provides a method for identifying a peptide or
15 fragment derived from a mammalian Tsg101 protein, which is effective in reducing production of retroviral particles, e.g., particles of a lentivirus, including but not limited to HIV-1 and HIV-2, Simian Immunodeficiency Virus (SIV). The method comprises identifying a peptide that binds to a peptide comprising the PTAP motif of a HIV Gag protein. In a preferred embodiment, the peptide comprising the PTAP motif is the peptide
20 of SEQ ID NO:4. In another preferred embodiment, the peptide comprising the PTAP motif is a HIV Gag protein. Any standard method known in the art for identifying peptide binding can be used.

Preferably, the portion of a coding sequence for a mammalian *tsg101* gene includes at least a portion of the UEV A domain of Tsg 101. The UEVA region spans amino acids 1
25 through 140 of a mammalian Tsg101.

In another preferred embodiment, the mammalian cells are primate cells. Mammalian 25 cells useful for practicing the invention include but are not limited to green monkey kidney cells, human HeLa cells, and human 293 cells. These and other mammalian cell lines are available from a variety of sources such as the American Type Culture
30 Collection (ATCC), Manassas, Va.

A mammalian *tsg101* gene may be obtained from any mammalian cell. For example, a human *tsg101* gene may be isolated from B cells or a B cell library (Durfee T., et al. 1993, *Genes Dev.* 7:555-569). Similarly, a mouse or other mammalian *tsg101* gene may be isolated from B cells or a B cell library. The nucleotide sequence and corresponding amino
35 acid sequence for a human *tsg101* gene and a mouse *tsg101* gene are known. See, e.g., Li, L.

et al. (1997) *Cell* 88:143-154, which is incorporated by reference herein. Relevant mouse and human *tsg101* genes are also disclosed in U.S. Patent Nos. 5,807,995; 5,892,016; 5,679,523; and 5,891,668, which are also incorporated by reference herein. The
5 aforementioned disclosures, as well as any other publications mentioned herein, are incorporated by reference as if fully set forth. The mouse Tsg101 protein is also set forth herein as SEQ ID NO:1. The human Tsg101 protein is set forth herein as SEQ ID NO:2.

Peptides derived from a mammalian Tsg101 protein may be obtained by truncating the 3', 5' or both 3' and 5' end of the *tsg101* coding sequence by genetic manipulation. *In vitro* mutagenesis is helpful for introducing convenient restriction sites. There are various
10 commercially available kits particularly suited for this application such as the T7-Gene *in vitro* Mutagenesis Kit (USB, Cleveland, OH) and the QuikChange Site Directed Mutagenesis Kit (Stratagene, San Diego, CA). Alternatively, PCR primers can be defined to allow direct amplification of a particular portion of a *tsg101* coding sequence. (See, e.g., Example 1). Preferably, the portion of the *tsg101* coding sequence corresponds to a portion
15 of the UEVA region. A portion of the coding sequence for a mammalian *tsg101* gene may be inserted into many different types of expression vectors. For example, the expression construct comprising a portion of the coding sequence for a mammalian *tsg101* gene may be any expression construct which functions in mammalian cells. In a more preferred embodiment, the coding sequence for a portion of a *tsg101* gene is placed in a vector which
20 encodes both monomers of a heterodimeric ecdysone-inducible receptor, that together with the hormone ecdysone or ecdysone analogue such as pronasterone, form a transcriptional activator complex that binds the promoter driving *tsg101* expression.

Expression vectors encoding different HIV genes are widely known and available. For example, *tgp-RRE-r* (Smith et al. 1990 *J. Virol.* 64:2743-2750) is an expression vector
25 comprising both the *gag* and *pol* coding regions. pCMV-*rev* is an expression vector comprising the HIV-1 *rev* gene. Rev is an HIV-1 encoded protein required for expression of *gag* and *pol*. In mammalian host cells, a number of expression systems using elements from viral and cellular DNA may be utilized for expressing a portion of a *tsg101* coding sequence and the HIV *gag*, *pol* and *rev* coding sequences.

30 Any known method may be used to quantify HIV particles released from the cells. Quantification of HIV particles released from the cells may be performed using different methods such as by p24 ELISA or by infectivity assay as described in Flint, S.J. *et al.*, 2000, *Principles of Virology: Molecular Biology, Pathogenesis and Control*, ASM Press, Washington, D.C. Virus particles may first be isolated from culture media or supernatant
35 by passing through a filter such as e.g., a 0.22 μ m filter followed by ultracentrifugation

through a 20% sucrose cushion. Following disruption of HIV particles with a suitable detergent such as e.g., 1% SDS, p24 protein may be separated by electrophoresis through a 12.5% SDS polyacrylamide gel, followed by Western blotting using antibodies specific to p24 such as anti-CA rabbit polyclonal antibody raised against a native form of the CA protein (Ehrlich, L.S., et al., 1990 *AIDS Res & Hum. Retro.* 6:1169-1175), or anti-CA mouse monoclonal antibody (NEN-DuPont).

In accordance with the present invention, there are provided Tsg101 peptides which comprise at least about four contiguous amino acids of a mammalian Tsg101 protein, which are effective in reducing HIV particle production and which are identified by a subject assay hereinbefore described. Preferably, a subject peptide comprises at least about 5 contiguous amino acids which are effective in reducing HIV particle production and which are identified by a subject assay hereinbefore described. Even more preferred is a peptide comprising at least about 6 contiguous amino acids and which is effective in reducing HIV particle production and which are identified by a subject assay hereinbefore described. In a most preferred embodiment, a subject peptide comprises at least about 7 contiguous amino acids. Thus, for example, a subject peptide may comprise at least about 4, preferably at least about 5, more preferably at least about 6, and most preferably, at least about 7 contiguous amino acids of the amino acid sequence as set forth in SEQ ID NO:1 or SEQ ID NO:2. Preferably, a subject peptide is derived from or includes part of the UEV A domain of a mammalian Tsg101 protein. As used herein, "peptide" refers to a fragment of a Tsg101 protein comprising at least about four amino acids. Thus, a peptide may comprise as little as about four amino acids of a mammalian Tsg101 protein. A subject peptide may also comprise anywhere from about four to about 140 contiguous amino acids from a mammalian Tsg101 protein. Amino acid sequences larger than about 140 amino acids are referred to herein as Tsg101 fragments. Additionally, analogs, homologs, fragments, chemical derivatives, and pharmaceutically acceptable salts of the subject peptides are included within the scope of the term "peptide". A subject peptide may also comprise a repetition of an amino acid unit.

By "analogs" is meant substitutions or alterations in the amino acid sequences of a subject peptide, which substitutions, or alterations, e.g., additions and deletions of amino acid residues, do not abolish the property of reducing HIV particle production or inhibiting Tsg101-Gag binding. Thus, an analog may comprise a peptide having a substantially identical amino acid sequence to a subject peptide and in which one or more amino acid residues have been conservatively substituted with chemically similar amino acids.

In addition to the recombinant DNA techniques described above for preparing a subject peptide, a peptide of the present invention may be synthesized by a number of known techniques. For example, a peptide may be prepared using the solid-phase synthetic technique initially described by Merrifield, in *J. Am. Chem. Soc.* 85:2149-2154 (1963).

- 5 Other peptide synthesis techniques may be found in M. Bodanszky et al. *Peptide Synthesis*, John Wiley & Sons, 2d Ed. (1976) and other references readily available to skilled artisans. A summary of polypeptide synthesis techniques can be found in J. Stuart and J. D. Young. *Solid Phase Peptide Synthesis*, Pierce Chemical Company, Rockford, Ill., (1984). Peptides may also be synthesized by solution methods as described in *The Proteins*, Vol. II, 3d Ed.,
- 10 Neurath, H. et. al., Eds., p. 105-236, Academic Press, New York, N. Y. (1976). Appropriate protective groups for use in different peptide syntheses are described in the above-mentioned texts as well as in J. F. W. McOmie, *Protective Groups in Organic Chemistry*, Pelenu Press, New York, N.Y. (1973).

- The peptides of the present invention might also be prepared by chemical or
- 15 enzymatic cleavage from larger portions of a mammalian Tsg101 protein or from an entire mammalian Tsg101 protein. The peptides of the present invention are preferably chemically synthesized by the Merrifield solid phase technique. In general, the method comprises the sequential addition of one or more amino acid residues to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid residue is
- 20 protected by a suitable, selectively removable protecting group. A different, selectively removable protecting group is utilized for amino acids containing a reactive side group such as lysine.

- The preferred method of solid phase synthesis entails attaching the protected or derivatized amino acid to an inert solid support through its unprotected carboxyl or amino
- 25 group. The protecting group of the amino or carboxyl group is then selectively removed and the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected is admixed and reacted under conditions suitable for forming the amide linkage with the residue already attached to the solid support. The protecting group of the amino or carboxyl group is then removed from this newly added amino acid residue,
- 30 and the next amino acid (suitably protected) is added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining terminal and side group protecting groups including the solid support are removed sequentially or concurrently to yield the final peptide.

- In still another aspect of the invention, there is provided a method for identifying a
- 35 peptide comprising at least four contiguous amino acids of the HIV-1 late (L) domain and

which peptide inhibits interaction between a Tsg101 protein and HIV Gag. The HIV-1 late L domain is made up of the amino acid sequence PTAPP (SEQ ID NO:3), which is also referred to as the PTAPP motif. Thus, a peptide identified by the method will have at least four contiguous amino acids of the PTAPP motif and may also include additional amino acids on either or both the amino terminal and carboxy terminal ends. Preferably, a peptide identified by the method will comprise at least seven amino acids, including the PTAPP motif. More preferably a peptide identified by the method will include at least four amino acids of the PTAPP motif and one or more amino acids which naturally flank the PTAPP motif. PTAPP-derived peptides may embody repetition of the five amino acid, PTAPP motif. The amino acids which flank the PTAPP motif are indicated below (L domain indicated in bold).

ALQSRPEPTAPPEES (SEQ ID NO:4).

A method for identifying a peptide comprising the HIV-1 Gagp6 late (L) domain and which peptide inhibits interaction between a Tsg101 protein and HIV Gag comprises the following steps. First, a peptide comprising the HIV-1 Gag or HIV-1 Gagp6 late (L) domain is immobilized on a solid support or surface such as a bead, plate, slide, or microtiter dish. Immobilization may be achieved utilizing specific anti Gag or HIV Gagp6 late domain antibodies or other chemical linkers such as, e.g., UV cross linkers. Both an experimental and control run are performed and thus, at least two separate solid supports or surfaces (i.e., a first and second solid support or surface) have the HIV-1 Gag or HIV-1 Gagp6 late (L) domain immobilized thereon (e.g., one column for the experimental run, and one column for the control run). A first (experimental) reaction mixture comprising labeled Tsg101 protein, buffer, and a peptide which comprises the HIV-1 Gagp6 late (L) domain is incubated at about 4C for a sufficient time. A second (control) reaction mixture comprising labeled TSG101 and a buffer is also incubated at about 4C for a sufficient time. Preferably, a sufficient time is about 60 minutes. The first (experimental) reaction mixture is added to the first solid support or surface and incubated in a liquid phase for a sufficient time at about 4C. Likewise, the second (control) reaction mixture is added to the second solid support or surface and incubated in a liquid phase for a sufficient time at about 4 C. Preferably, a sufficient time is about 60 minutes. Next, the first solid support or surface is separated from the liquid phase to obtain a first solid phase. Likewise, the second solid support or surface is separated from the liquid phase to obtain a second solid phase. The amount of labeled Tsg 101 in the first solid phase and in the second solid phase is then

determined. A decrease in the amount of labeled TSG101 in the first solid phase when compared to the amount of labeled Tsg101 in the second solid phase correlates with the identification of a peptide comprising the HIV-1 Gagp6 late (L) domain, which peptide inhibits interaction between a Tsg101 protein and HIV Gag. Appropriate antibodies which
5 may be linked to the immobilized peptide comprising the HIV-1 Gag or HIV-1 Gagp6 late (L) domain include anti-p6 rat monoclonal antibody (ABI) and anti Gag CA (NEN-Dupont).

The invention also provides a method for identifying a peptide comprising a PTAP motif, which modulates or inhibits interaction between a Tsg101 and a PTAP or related motif of a viral protein which the Tsg101 interacts with. The peptide can be used for
10 reducing production of virus particles, e.g., particles of a lentivirus, including but not limited to HIV-1 and HIV-2, Simian Immunodeficiency Virus (SIV). The method comprises identifying a peptide comprising a PTAP motif which binds to a TSG101 protein. In a preferred embodiment, the peptide comprises the sequence of SEQ ID NO:4. The method is also applicable for identifying other molecules that modulate or inhibit interaction
15 between a Tsg101 and a PTAP or related motif of a viral protein which Tsg101 interacts with, e.g., a PTAP motif of a HIV Gag, e.g., by binding to the active site of the Tsg101 or the viral protein. Therefore, in another aspect of the invention, methods for identifying molecules that modulate or inhibit interaction between a Tsg101 and a PTAP or related motif of a viral protein are provided. Any standard method known in the art for identifying
20 peptide binding can be used.

Tsg101 may be labeled with a fluorescer, enzyme, chemiluminescer, photosensitizer, suspendable particles, or radioisotope using any of the common commercially available signal producing systems. Alternatively, Tsg101 may be labeled with a tag such as e.g., an antibody, a 6His tag, GST tag, or influenza virus epitope (HA tag). The Tsg101 protein for
25 use in the assay described above may be from a mouse, human or other mammalian protein.

Preferably, a peptide identified by the assay causes at least about a three-fold reduction in Tsg101-HIV Gag interaction. Any of the peptides of the present invention may be used as a therapeutic composition for reducing HIV particle formation or for inhibiting Tsg101-HIV Gag interaction. Preferably, the therapeutic composition is a pharmaceutical
30 composition. Thus, the peptides of the present invention may be administered preferably to a human subject as a pharmaceutical composition containing a therapeutically effective dose of at least one of the peptides according to the invention, together with one or more pharmaceutically acceptable carriers or diluents. The term "therapeutically effective amount" means a dose needed to reduce HIV particle formation or other retrovirus
35 formation or inhibit Tsg101-HIV Gag interaction, depending on mode of action of a subject

peptide. Such mode of action is determined by an appropriate assay as hereinbefore described.

When administered intravenously, the peptide compositions may be combined with other ingredients, such as carriers, and/or diluents and/or adjuvants. Typical carriers include
5 a solvent or dispersion medium containing, for example, water buffered aqueous solutions, ethanol, polyols such as glycerol, propylene glycol, polyethylene glycol, suitable mixtures thereof, surfactants or vegetable oils. Isotonic agents such as sugars or sodium chloride may be incorporated in the subject compositions. The peptides may also be covalently attached to a protein carrier, such as albumin, so as to minimize clearing of the peptides from the
10 blood. There are no limitations on the nature of the other ingredients, except that they must be pharmaceutically acceptable, efficacious for their intended administration and should not degrade the activity of the active ingredients of the compositions. The peptide compositions of the invention may also be impregnated into transdermal patches or contained in subcutaneous inserts, preferably in liquid or semi-liquid form so that a therapeutically
15 effective amounts of one or more subject peptides may be time-released into a subject.

When the peptides of the invention are administered orally, the pharmaceutical compositions thereof containing an effective dose of the peptide may also contain an inert diluent such as an assimilable edible carrier and the like, be in hard or soft shell gelatin capsules, be compressed into tablets, or may be in an elixir, suspension, syrup or the like.
20 The subject peptides are thus compounded for convenient and effective administration in pharmaceutically effective amounts with a suitable pharmaceutically acceptable carrier in a therapeutically effective dose.

The precise therapeutically effective amount of peptides to be used in a method of treating a patient infected with HIV will be determined by the practitioner based on the age,
25 weight and/or gender of the subject, severity of the disease state, diet, time and frequency of administration, drug combination(s), and/or reaction sensitivities. It can generally be stated that the peptides should preferably be administered in an amount from about 0.1 to 100,000 micrograms as a dosage amount, up to a total dose of about 1 gram, depending on the route of administration. Since the peptide compositions of the invention will eventually be
30 cleared from the bloodstream, re-administration of the compositions is indicated and preferred.

6. EXAMPLES

The following examples are presented by way of illustration of the present
35 invention, and are not intended to limit the present invention in any way.

6.1. EXAMPLE 1

In this example, HIV-1 Pr55^{Gag} was used as bait in a yeast 2-hybrid screen and identified Tsg101, the product of a mammalian tumor susceptibility gene, Tsg101 (Li, L. & Cohen, S. N., 1996, *Cell* 85, 319-329), as a cellular protein that interacts with HIV-1 Gag.

5 The example demonstrates that Tsg101 interacted specifically with the p6 region of HIV-1 Gag both in vitro and in the cytoplasm of transfected cells. Two highly conserved Pro residues in the L domain within p6 were critical for Tsg101 binding. Moreover, the altered Ub-binding site in the UEV domain in Tsg101 as well as other residues unique to Tsg101 were determinants of interaction with Gag. These results implicate a specific component of

10 the cellular trafficking machinery in virus budding and maturation.

Ubiquitination appears to be involved in virus particle release from infected cells. Free ubiquitin (Ub), as well as Ub covalently bound to a small fraction of p6 Gag is detected in mature human immunodeficiency virus particles. The p6 region in the Pr55^{Gag} structural precursor polyprotein binds to Tsg101, a putative Ub regulator that is involved in

15 trafficking of plasma membrane-associated proteins. Tsg101 was found to interact with Gag in (i) a yeast 2-hybrid assay, (ii) *in vitro* co-immune precipitation using purified Pr55^{Gag} and rabbit reticulocyte lysate-synthesized Tsg101, and (iii) *in vivo* in the cytoplasm of COS cells transfected with *gag*. The PTAPP motif (or late (L) domain) within p6, which is required for release of mature virus from the plasma membrane, was the determinant for

20 binding Pr55^{Gag}. The N-terminal region in Tsg101, which is homologous to the Ubc4 class of Ub-conjugating (E2) enzymes, was the determinant of interaction with p6. Mutation of Tyr110 in 101, present in place of the active site Cys that binds Ub in E2 enzymes, and other residues unique to Tsg101 impaired p6 interaction indicating that features that distinguish Tsg101 from active E2 enzymes were important for binding the viral protein.

25 The results link L domain function in HIV to the Ub machinery and a specific component of the cellular trafficking apparatus.

Materials and Methods

30 **Plasmid construction.** Oligonucleotides and procedures used for PCR and mutagenesis to construct Pr55^{Gag} pl-p6, and Tsg101 GAL4-hybrids for expression in yeast; human Tsg101 for in vitro expression; and Pr55^{Gag Δp6} for expression in mammalian cells are described hereinbelow and in Table 1.

Pr55^{Gag} and pl-p6 hybrids for expression in yeast. HIV-1 sequences were amplified

35 by PCR using plasmid gpVI as template to synthesize inserts for ligation into yeast

expression vectors *pGBT9* or *pMA424* and *pGAD424*. *pgpVI* encodes Pr55^{Gag} and part of Pol, and an inactivating mutation in the catalytic site of PR (D₂₅A) within Pol. The 5' end primer, oligo no.1 in Table 1, and the 3' end primer (no.2) were used to synthesize a PCR fragment of full-length gag. The primers annealed to nt 333 of *gag* and the end of the PR coding region, respectively. BamHI and BglII sites were engineered in the insert for ligation into *pGBT9* and *pGAD424*. For the MA-CA insert, we used the same 5' end primer (no.1) and a 3' end primer (no.3) that annealed at position 1421 in the *gag* gene at the C-terminus of CA. These oligonucleotides contained the restriction sites BglII and BamHI respectively. For the CA-p2-NC insert, a 5' primer (no.4) that annealed to the *gag* gene at position 730 at the N-terminus of CA and a 3' end primer (no.5) complementary to position 1673 of the *gag* gene at the C-terminus of p1 were used. The PCR product was cut with BglII and ligated into the vector at that site. For the p1-p6 insert, a 5' end primer (no.6) that annealed to the *gag* gene at position 1636 and a 3' end primer (no.7) that annealed at position 2116 in the *pci* gene were used. The insert obtained spanned the p1-p6 region of Gag in the *gag* frame and the first ninety-nine amino acids of the *pci* frame. It lacked the frameshift site located in the p1 region upstream of nucleotide 1636 so that only expression of the *gag* frame was obtained. Both oligonucleotides contained BglII sites. The Pr55^{Gag} Δ p6 insert was created by restriction digestion at the unique BglII site. *gag* sequences were ultimately placed in the *pGBT9* vector background by transferring inserts from *pMA424* to *pGBT9* using the BamHI and SalI sites. Deletions and site-directed mutations in *pGBT9*-p1-p6 were created by mutagenesis using the Gene Editor (Promega). Mutagenic oligonucleotides (nos.8-15) were used according to the manufacturer's protocol. Mismatch regions, introduced for point mutations leading to amino acid changes or deletion mutations are underlined or indicated by "/", respectively. Double mutations were created using a second selection oligonucleotide (no.13) that obliterates a SnaBI restriction site within the vector. Resistance to SnaBI digestion selected for the mutant plasmids. The double mutant K_{487, 493}R was synthesized using the second selection oligonucleotide and the K₄₈₇R mutagenic oligo (no.11) with the mutant K₄₈₇R as the template. The double mutant Δ PTAPP_{1,2} was synthesized using the second selection oligonucleotide and the Δ PTAPP₂ mutagenic oligo (no.9), with Δ PTAPP₁ as the template. Point mutations in the second Δ PTAPP₁ motif were synthesized from the mutant Δ PTAPP₁ using the second selection oligo and oligos nos.14 and 15, as indicated in Table 1.

Tsg101 hybrids for expression in yeast. Tsg101, as isolated from a B cell library in a pACT vector, was used as template for PCR of inserts for the yeast two-hybrid system. The inserts were engineered with EcoRI and SalI sites for ligation into *pGAD424*. For the

wild-type tsg101 insert, the forward (5' end) primer (no. 16) was complementary to the beginning of the coding region of Tsg101. The reverse (3' end) primer was no. 17. Truncations at the 3' end were introduced using the tsg101 forward primer (no. 16) and reverse primers nos. 18-20, all with translational stop codons and Sall sites introduced at the downstream terminus of the gene. Truncations at the 5' end were introduced by using the tsg101 reverse primer (no. 17) and forward primers nos. 21-23, each with an EcoRI site introduced before the 5' terminus of the sequence. Site-directed mutations in tsg101 were created using the Gene Editor and oligos nos. 24-28.

In vitro expression. Human tsg101 was subcloned into the BamHI site of the pET3a vector under the control of the T7 promoter (Novagen). The insert was produced by PCR using pACT-tsg101 as template with 5' primer no. 29, which anneals at the position corresponding to amino acid 10 in the protein, and 3' primer no. 30, which anneals to the C-terminus of the coding region.

Pr55^{GagAp6} for expression in mammalian cells. pgp-RRE-r was used as a template to synthesize three point mutations in the first codon of p6 by PCR, thereby converting it into a stop (ochre) codon. The 5' primer (no. 31) anneals to nucleotides 1637–1687 of BH10 gag and includes a unique BglII site. The 3' primer (no. 32) anneals to nucleotides 2655–2682 in the pol gene downstream of a unique EcoRV site. The pgp-RRE-r plasmid and the fragment produced from PCR were cut with BglII and EcoRV, and the desired fragments were ligated.

All constructs were confirmed by sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA) according to the manufacturer's instructions.

See, e.g., Ehrlich, L. S., Fong, S., Scarlata, S., Zybarth, G. & Carter, C., 1996, Biochemistry 35, 3933–3943; Durfee, T., Becherer, K., Chen, P. -L., Yeh, S. -H., Yang, Y., Kilburn, A. E., Lee, W.-H. & Elledge, S. J., 1993, Genes Dev. 7, 555–569; Smith, A. J., Cho, M. I., Hammariskjold, M. L. & Rekosh, D., 1990, J. Virol. 64, 2743–2750; Ratner, L., Hasteline, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., et al., 1985, Nature (London) 313, 277–284.

Table 1 Oligonucleotides Used in PCR and Mutagenesis

<u>Insert</u>	<u>Template</u>	<u>#</u>	<u>Sequence</u>
gag	pgpVI	1	GGCTAGAAGGATCCGGATGGGTGCGAGAGCGTCAG (SEQ ID NO:1)
		2	GAAGATCTATTAGAAGTTTAAAGTGC (SEQ ID NO:6)
		3	GAAGATCTCACTACAAAACCTCTTGCC (SEQ ID NO:7)
MA-CA		1	

CA-p2-NC		4	GGAAGATCTCCCTATAGTGCAGAACATCC (SEQ ID NO:8)
		5	CGGGATCCTTCCCTGGCCTTCCC (SEQ ID NO:9)
p1-p6		6	GGGAAGATCTGGCCTTCC (SEQ ID NO:10)
		7	GAAGATCTATTAGAAGTTTAAAGTGC (SEQ ID NO:11)
5	PTAPP ₁	pGBT9-p1p6	8 CAGAGCAGACCAGAG/TTTCTTCAGAGCAGACC (SEQ ID NO:12)
	PTAPP ₂		9 CAGAGCAGACCAGAG/GAAGAGAGCTTCAGG (SEQ ID NO:13)
	K ₄₈₇ R (AAG)		10 CCCCTCAGAGCCAGGAGCC (SEQ ID NO:14)
	K ₄₉₃ R (AAG)		11 GCCGATAGACAGGGAAGTGTATC (SEQ ID NO:15)
	(LXX) ₄		12 CCGATAGACAAGGAA/AACGACCCCTCGTCAC (SEQ ID NO:16)
K _{487, 493} R		pGBT9-p1p6 _{K487R}	11
(TAC)			13 CATTTCCTATATGTAGTATATAG (SEQ ID NO:17)
10	PTAPP _{1,2}	pGBT9-p1p6	9
	PTAPP ₁		13
P ₇ A		pGBT9-p1p6	13
		PTAPP ₁	
(CCA)			14 GACCAGAGGCAACAGCC (SEQ ID NO:18)
P _{10,11} A			13
15	(CCA,CCA)		15 CAGAGCCAACAGCCGAGCATTCTTCAGAGC (SEQ ID NO:19)
	tsg101	pACT-tsg101	16 GAATTCATGGCGGTGTCTGGAGAGC (SEQ ID NO:20)
			17 GTCGACTCAGTAGAGGTCACTGAG (SEQ ID NO:21)
N-155			16
			18 GTCGACTCATGCCTGGTATGGCGG (SEQ ID NO:22)
N-215			16
			19 GTCGACTCAGGGACCAACAGTGGTCAC (SEQ ID NO:23)
20	N-311		16
			20 GTCGACTCAGTTTTTCAGACTGATTTTCC (SEQ ID NO:24)
139-C			17
			21 GAATTCCTCCAGTCTTCTCTCGTCC (SEQ ID NO:25)
241-C			17
			22 GAATTCGGATGAAGGAGGAAATGGATCG (SEQ ID NO:26)
311-C			17
25			23 GAATTCAATGATATCGATGAAGTTATCATTCCC (SEQ ID NO:27)
	K ₁₀₈ A (AAG)	pGAD-tsg101	24 GTTGATGCAAAATGGGGCGATATATCTTCC (SEQ ID NO:28)
	Y ₁₁₀ W (TAT)		25 AATGGGAAGATATGGCTTCCTTATCTAC (SEQ ID NO:29)
	Y ₁₁₃ V (TAT)		26 GGGAAGATATATCTTCTGCTCTACATGAATGG (SEQ ID NO:30)
	W ₁₁₇ A		27 CCTTATCTACATGAAGCGAAACACCCACAG (SEQ ID NO:31)
(TGG)			
30	K ₁₁₈ A (AAA)		28 CTACATGAATGGGCACCCACAGTCAG (SEQ ID NO:32)
	tsg101	pACT-tsg101	29 GGATCCATGGTGTCCAAGTAC (SEQ ID NO:33)
			30 GGATCCTCAGTAGAGGTCACTGAG (SEQ ID NO:34)
gag p6			31 GGAAGATCTGGCCTTCTACAAGGGAAGGCCAGGGAATT (SEQ ID NO:35)
			32 CCATGTATTGATAGATAACTATGTCTG (SEQ ID NO:36)

2-hybrid assay. The PR55^{Gag}-Tsg101 interaction was identified by a yeast 2-hybrid screen using a human B cell library (Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A. E., Lee, W.-H. & Elledge, S. J., 1993, *Genes Dev.* 7, 555-569). Vectors pGBT9 and pGAD424 encoding PR55^{Gag} or Tsg101 sequences as GAL4 activation and binding domain fusion proteins were transformed into *Saccharomyces cerevisiae* Y153 using procedures previously described (Bartel, P. L. & Fields, S., 1995, *Methods Enzymol.* 254,241-263). Briefly, interactions were detected using a selection for Trp and Leu prototrophy followed by quantitative assay for *LacZ* activation. True positives were confirmed by demonstrating that they failed to interact with vectors carrying no insert or vectors carrying nonspecific genes (lamin). Proteins were identified after automated sequencing and matching the DNA to a protein sequence in the database. Mapping of the interacting domain was performed using vectors encoding the DNA-binding or activation domain of the yeast GAL4 transcriptional activator protein fused to truncations, deletions or point mutations of the proteins. Interactions were tested in both orientations: The text describes the interactions of the GAL4 DNA-binding domain-Gag or -p1-p6 fusion proteins with the GAL4 activation domain-Tsg101 fusion protein. Expression of all GAL4 fusions was checked by analysis of yeast cell extracts by SDS-gel electrophoresis and Western blotting with an antibody directed against the GAL4 binding domain (Upstate Biochemical) and GAL4 transactivation domain (Santa Cruz).

Cell culture, transfection, and preparation of cytoplasmic extracts. COS-1 cells were cultured in DMEM supplemented with fetal bovine serum to 60% confluency at 37°C. The cells were transfected using the FuGene 6 reagent (Roche) according to the instructions of the manufacturer. At 48 hr post-transfection, the cells were harvested into the media and collected by centrifugation. The pelleted cells were washed with cold PBS, allowed to swell in hypotonic buffer (10 mM Tris, pH 7.4, 1 mM MgCl₂; 4°C) containing protease inhibitors, and disrupted with a Dounce homogenizer (type B pestle). The total lysate was spun for 10 min at 10,000 x g at 4°C to remove unbroken cells, nuclei, and mitochondria.

Immune capture assays. For *in vitro* assay of Tsg101-Gag interaction, Tsg101 was expressed in rabbit reticulocyte lysate (RRL) from a pET3a-*tsg101* construct in the presence of [³⁵S]-Met (DuPont NEN) using the TNT T7 Quick Coupled Transcription/Translation System (Promega). Recombinant Pr55^{Gag} produced using the T7 RNA polymerase promoter and containing aa 1-10 of T7 gene 10 at the N-terminus, was purified from an expression strain of *E. coli* (BL21-DE3) as previously described (Ehrlich, L. S., Fong, S., Scarlata, S.,

Zybarth, G. & Carter, C., 1996, *Biochemistry* 35, 3933-3943). Protein A agarose beads (Pierce), pre-washed with non-denaturing buffer (25mM Tris, pH 7.4, 150mM NaCl, 0.5mM MgCl₂, 1mM CaCl₂, 1% IGEPAL (Sigma)) containing protease inhibitors (Roche) were incubated with the appropriate antibody, washed again, and then pre-incubated with
 5 Gag. Radiolabeled Tsg101 was then added, and the mixture incubated further at 4°C in a rotating device for 60 min. The beads were washed, suspended in SDS-PAGE loading buffer and heated at 95°C for 5 min. Cytoplasmic extracts prepared as described above were also examined for Tsg101-Gag interaction using the same procedure, except that the extract and the antibody-coated Protein A beads were maintained in 10 mM Tris, pH 7.4, 1
 10 mM MgCl₂.

Protein detection. Proteins were separated by electrophoresis through a 12.5% SDS polyacrylamide gel. For detection of radiolabeled Tsg101 after electrophoresis, gels were fixed, incubated for 30 min in EN³HANCE (DuPont NEN) autoradiography enhancer for
 15 gel fluorography and dried. Radioactive bands were visualized using imaging film (BioMax, Kodak). Gels with non-radioactive samples were transferred to nitrocellulose and analyzed by Western blotting. The following antibodies, as specified in the text, were used: anti-capsid (CA)₁ and -CA₂ (rabbit polyclonal antibodies raised against native and denatured forms of the CA protein, respectively; Ebbets-Reed, D., 1996, in *Molecular Microbiology*,
 20 State University of New York at Stony Brook, Stony Brook, NY, pp. 209; Ehrlich, L. S., Agresta, B. E. & Carter, C. A., 1992, *J. Virol.* 66,4874-4883); anti-CA3 (mouse monoclonal antibody, NEN-DuPont); anti-p6 (rabbit polyclonal against the C-terminal 16 amino acids, S. Campbell, NCI-FCRDC); anti-nucleocapsid (NC, goat polyclonal, A. Rein, NCI-FCRDC); anti-T7 (Novagen); anti-Tsg101₁ (monoclonal, Santa Cruz); antiTsg101₂
 25 (rabbit polyclonal, obtained from S. Cohen, Stanford U.). Proteins were visualized by chemiluminescence with Lumi-Light (Roche).

Results

30 **Pr55^{Gag} interacts with Tsg101 in the 2-hybrid assay.** HIV-1 Pr55^{Gag} was used as bait in the yeast 2-hybrid screen (Bartel, P. L. & Fields, S., 1995, *Methods Enzymol.* 254,241-263) to identify interacting proteins encoded in a cDNA library derived from human B cells (Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A. E., Lee, W.-H. & Elledge, S. J., 1993, *Genes Dev.* 7, 555-569). Approximately 2 million transformants were
 35 screened and 2 positives were isolated. Sequencing and matching to recorded entries in the

GeneBank database identified one interacting partner as cyclophilin B, which had been previously found to interact with Pr55^{Gag} (Luban, B., Bossolt, K. L., Franke, E. K., Kalpana, G. V. & Goff, S. P., 1993, *Cell* 73, 1067-1078). The other interacting protein was identified as the product of the human tumor susceptibility gene, Tsg101 (Li, L. & Cohen, S. N., 1996, *Cell* 85, 3 19-329; Li, L., Li, X., Francke, U. & Cohen, S. N., 1997, *Cell* 88, 143-154).

Identification of the region in PrSSGat that interacts with Tsg101. To localize the region of Pr55^{Gag} required for interaction with Tsg101, plasmids encoding N- or C-terminally-deleted-Pr55^{Gag} fused to the DNA-binding domain of the yeast GAL4 protein were tested for *LacZ* reporter gene activation using the 2-hybrid assay. Western analysis, using an antibody against the GAL4 DNA binding domain in the fusion proteins showed that the mutated and wild-type proteins were all expressed at comparable levels. As shown in FIG. 1A, the signal was lost upon deletion of the p1-p6 region of Pr55^{Gag} but was retained in plasmids encoding p1-p6. Thus, interaction with Tsg101 was determined by elements within p1-p6 of Pr55^{Gag}.

The p6 region contains the conserved motifs P₇T/SAP₁₀P₁₁ (numbering within p6 domain) and a repeating Leu sequence (LXX)₄. The (LXX)₄ motif is a critical determinant for Vpr binding although it may not interact directly (see, e.g., Frankel, A. D. & Young, J. A., 1998, *Annu. Rev. Biochem.* 67, 1-25). The PTAPP motif, or late (L) domain and, in particular, Pro_{10,11}, is the determinant of mature virus release during the final stages of assembly (Göttlinger, H. G., Dorfman, T., Sodroski, J. G. & Haseltine, W. A., 1991, *Proc. Natl. Acad. Sci. USA* 88, 3195-3199; Huang, M., Orenstein, J. M., Martin, M. A. & Freed, E. O., 1995, *J. Virol.* 69, 6810-6818). The PTAPP motif overlaps a region (P₅XP₇) that is critical for efficient packaging of *pol* gene products into the assembled virus particle (Dettenhofer, M. & Yu, X. F., 1999, *J. Virol.* 73, 4696-4704). The p6 region also contains Lys residues that are substrates for ubiquitination (Ott, D. E., Coren, L. V., Copeland, T. D., Kane, B. P., Johnson, D. G., Sowder, R. C., 2nd, Yoshinaka, Y., Oroszlan, S., Arthur, L. O. & Henderson, L. E., 1998, *J. Virol.* 72, 2962-2968). To determine whether any of the above conserved motifs were important for Tsg101 binding, deletion and point mutations were engineered into a plasmid encoding the p1-p6 region of Pr55^{Gag} and protein-protein interactions were measured using the 2-hybrid assay. The p6 region from the pBH10 clone used for these studies contained 2 copies of the PTAPP motif (Ratner, L., Haseltine, W., Patarca, R., Livak, K. 3., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K. & et al., 1985, *Nature* 313, 277-284) and each of these was deleted independently. Deletion of the first PTAPP motif (aa 455-459, numbering

- within Gag) reduced β -galactosidase activity to a level that was ~50% that of wild-type protein binding (Fig. 1B). Deletion of the second PTAPP motif (aa 467-471), which is conserved among all HIV strains and most lentiviruses (Myers, G., Korber, B., Wain-Hobson, S., Smith, R. F. & Pavlakis, G. N., 1995, Los Alamos National Laboratory, Los Alamos, NM), reduced β -galactosidase activity to ~25% that of wild-type protein binding. Deletion of both motifs (aa 455 - 459 and 467 - 471) reduced β -galactosidase activity to the level obtained when the vector lacked an insert (<0.3%). Deletion of the first PTAPP-motif combined with substitution of Ala for Pro₇ in the second motif reduced enzyme activity to 25% that of wild-type. In the same context, substitution of Ala for Pro_{10,11} blocked the interaction completely. Substitution of Arg for either or both of the Lys residues that serve as substrates for Ub modification (Ott, D. E., Coren, L. V., Copeland, T. D., Kane, B. P., Johnson, D. G., Sowder, R. C., 2nd, Yoshinaka, Y., Oroszlan, S., Arthur, L. O. & Henderson, L. E., 1998, *J. Virol.* 72,2962-2968) gave wild-type β -galactosidase activity indicating that the Ub substrates in p6 are not required for Tsg101 recognition.
- Deletion of the (LXX)₄ repeat motif reduced β -galactosidase activity to ~55% of the wild-type level. The same reduction was obtained when the first PTAPP motif was removed in its entirety. The more deleterious impairment caused by deletion of the entire second highly conserved PTAPP motif or by mutation of Pro_{10,11} in this motif suggest that it is the major determinant of Tsg101 binding.

20

- Tsg101 binds to Pr55^{Gag} in vitro.*** To confirm and extend our observations with the 2-hybrid assay, *in vitro* co-immune precipitation studies were conducted using unlabeled bacteriophage T7 protein-tagged recombinant Pr55^{Gag} expressed in bacteria (Ehrlich, L. S., Fong, S., Scarlata, S., Zybarth, G. & Carter, C., 1996, *Biochemistry* 35, 3933-3943.) and [³⁵S]-Met-labeled mouse or human Tsg101 expressed in RRL. Mouse (obtained from S. Cohen, Stanford U.) and human Tsg101 (from the B cell library) are 94% identical and were used interchangeably in these assays. The radiolabeled protein shown in Fig. 2A (lane 1) migrated at the molecular weight expected for Tsg101(391 amino acids, ~43 kDa). The protein was detected consistently as a doublet, perhaps due to internal initiation at Met₁₀.
- The protein was also sometimes detected as a doublet in cytoplasmic extracts. Tsg101 was captured by Protein A-coated beads on which antibodies directed against the T7 tag, CA, or p6 domains had been immobilized and pre-incubated with Pr55^{Gag} (lanes 3, 7, 8, and 9). Beads not preincubated with Gag (lanes 2, 4, 5, 6) or preincubated with Gag in the absence of antibody did not capture Tsg101. The anti-p6 antibody recognizes an antigenic site in the C-terminal 16 residues of the protein (including the (Leu-X-X)₄ repeat). The demonstration

- that the Pr55^{Gag} bound to this anti-p6 antibody was still able to capture Tsg101 indicates that the Tsg101 binding region in p6 was exposed. This is consistent with the 2-hybrid assay result, which showed that the C-terminal half of the p6 region was not a Tsg101 binding site. In competition assays, addition of a 5-fold molar excess of a peptide containing the
- 5 PTAPP motif (ALQSRPEPTAPPEES) caused a 2.2-fold reduction in Tsg101 capture by Pr55^{Gag}. The limited solubility of the peptide precluded testing at higher concentrations. In contrast, no change was detected with a 5-fold molar excess of a peptide containing the mutant LIAPP sequence, indicating that the effect of the PTAPP motif was specific. Western analysis with an anti-CA monoclonal antibody confirmed the presence of
- 10 full-length Pr55^{Gag} on the beads coated with the anti-T7, -CA and -p6 antibodies that captured Tsg101 (Fig. 2B, lanes I - 4). The results of the immune capture assay demonstrate that Tsg101 interacts specifically and stably with Pr55^{Gag} *in vitro* through interaction with the L domain.
- 15 ***Tsg101 binds Pr55^{Gag} in vivo.*** To determine if Tsg101 and Gag associate during viral assembly, COS cells expressing Gag were examined for Tsg101-Gag complexes by co-immune precipitation assays. Plasmid *pgp*-RRE-r expresses the HIV-1 Gag and Gag-Pol polyproteins as well as Vif under the control of the SV40 late promoter (Smith, A. J., Cho, M. I., Hammarskjold, M. L. & Rekosh, D., 1990, *J. Virol.* 64, 2743-2750). Expression
- 20 requires the Rev protein, which is provided in trans by expression of pCMV-*rev* (Smith, A. J., Cho, M. I., Hammarskjold, M. L. & Rekosh, D., 1990, *J. Virol.* 64, 2743-2750). To ensure the specificity of the Tsg101-Gag interaction in the cytoplasm, a Gag mutant that lacked the p6 domain was included. Cytoplasmic extracts prepared from cells transfected with *rev* alone, *rev*, *gag*, and *pol*, or *rev*, *gag* Δ p6, and *pol* were incubated with anti-Tsg101
- 25 mouse monoclonal antibody and the immune-precipitate was examined for Gag-related proteins by Western blotting with a goat polyclonal antibody against the NC domain. First, the total cytoplasmic extract was examined (Fig. 3A). The anti-NC antibody recognized Pr55^{Gag}, an 18 kDa NC-related protein, and NCp7 in the extract prepared from cells expressing the wild-type Gag protein (lane 2). The 18 kDa band was identified as NC-p6
- 30 based on its reactivity with both anti-NC and anti-p6. The anti-NC antibody also recognized NCp7 and a protein that migrated at ~49 kDa in the extract prepared from cells expressing the mutant (lane 3). The latter is the molecular weight expected for the Pr55^{Gag} Δ p6 precursor protein. Consistent with this conclusion, the 49 kDa protein was not detected in extracts prepared from cells expressing Rev alone (lane 1) or the wild-type Gag
- 35 protein (lane 2). Immune-precipitation using a monoclonal antibody against Tsg101

precipitated the wild-type Gag precursor and the NC-p6 protein, but not NCp7 (Fig. 3B, lane 2) or Gag lacking the p6 domain (lane 3). Re-probing the same blot with another anti-Tsg101 antibody confirmed the presence of the cellular protein in the immune-precipitates of all three extracts (lanes 4-6). In a reciprocal experiment, anti-CA antibody co-immune-precipitated Tsg101 from extracts of cells expressing Gag (Fig. 3C, lane 2) but not extracts expressing Rev alone (lane 1). Re-probing the same blot with another anti-CA antibody confirmed the presence of Gag on the beads that captured Tsg101 (lane 4). Immune precipitation with an irrelevant antibody (rabbit anti-mouse IgG) did not precipitate Gag or Tsg101. The results indicate that the interaction between Tsg101 and Gag occurs in the cytoplasm of cells containing the viral protein and demonstrate that the p6 region of the Gag protein specifically directs the interaction.

Identification of the region in Tsg101 that interacts with Pr55^{Gag}. The Tsg101 protein contains an N-terminal E2-like (UEV) domain with homology to the Ub-conjugating (Ubc) 4 subgroup of E2 enzymes (Koonin, E. V. & Abagyan, R. A., 1997, *Nat. Genet.* 16,330-331; Ponting, C. P., Cai, Y.-D. & Bork, P., 1997, *J. Mol. Med.* 75,467-469). It also contains central Pro-rich and coiled-coil regions, and a C-terminal steadiness box (SB) that controls its steady state level (Fig. 4; Feng, G. H., Lih, C. J. & Cohen, S. N., 2000, *Cancer Res.* 60, 1736-1741). Based on recent findings implicating Ub in Gag assembly (Ott, D. E., Coren, L. V., Copeland, T. D., Kane, B. P., Johnson, D. G., Sowder, R. C., 2nd, Yoshinaka, Y., Oroszlan, S., Arthur, L. O. & Henderson, L. E., 1998, *J. Virol.* 72,2962-2968; Schubert, U., Ott, D. E., Chertova, E. N., Welker, R., Tessmer, U., Princiotta, M. F., Bennink, J. R., Krausslich, H. G. & Yewdell, J. W., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13057-13062; Strack, B., Calistri, A., Accola, M. A., Palu, G. & Gottlinger, H. G., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13063-13068; Vogt, V. M., 2000, *Proc. Natl. Acad. Sci. USA* 97, 12945-12947; Ott, D. E., Coren, L. V., Chertova, E. N., Gagliardi, T. D. & Schubert, U., 2000, *Virology* 278, 111-121; Patnaik, A., Chau, V. & Wills, J. W., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13069-13074), it was of interest to determine which region of Tsg101 was recognized by the viral protein. To investigate this, the yeast 2-hybrid system was used to localize the region in Tsg101 required for interaction with Pr55^{Gag}. Plasmids encoding N- or C-terminally-truncated Tsg101 fused to the activation domain of the GAL4 protein were assayed for the ability to bind p1-p6 fused to the GAL4 DNA-binding domain. As shown in Figure 4A, assessment of reporter gene activation indicated that p6-Tsg101 interaction was maintained in fragments that retained the N-terminal 155 amino acids, but lost in the mutants that lacked this region, suggesting that the N-terminal region was the minimal

determinant of binding. Western analysis showed comparable expression of the interacting and non-interacting fragments, thus supporting this conclusion. Curiously, the N-terminal 155-, 215-, and 311- residue fragments bound p6 comparably in a qualitative assay, but the 311-residue fragment interacted to a significantly greater extent in the quantitative assay

5 (Figure 4A). This difference did not reflect a binding site downstream of aa155 since a fragment extending from aa139 to the C-terminus of Tsg101 failed to interact. Moreover, no binding was detected in *in vitro* capture assays using a hybrid protein comprised of GST fused to Tsg101 residues 167-374. Optimal presentation of the p6 binding region in the N-terminal 155 residue fragment may require downstream sequences. In this regard, Tsg101

10 resembles class II E2 enzymes with a conserved catalytic core domain of ~150 residues and an extra C-terminal extension attached to this core domain that is speculated to play a role in substrate recognition (Jentsch, S., Seufert, W., Sommer, T. & Reins, H.A., 1990, Trends Biochem. Sci. 15, 195-198; Li, L. Liao, J., Ruland, J., Mak, T.W. & Cohen, S.N., 2001, *Proc. Natl. Acad. Sci. USA* 98, 1619-1624).

15 In E2 enzymes including Ubc4, Ub is conjugated to an active site Cys. In Tsg101, Tyr replaces this Cys residue. However, 8 of 14 residues flanking this Tyr are conserved in both Tsg101 and Ubc4 (Koonin, E. V. & Abagyan, R. A., 1997, *Nat. Genet.* 16,330-331; Ponting, C. P., Cai, Y.-D. & Bork, P., 1997, *J. Mol. Med.* 75,467-469; Fig. 4B). To determine whether recognition by the p6 domain was due to amino acids unique to Tsg101,

20 point mutations were engineered into the active site homologue in the full-length protein and interaction with p6 was tested in the 2-hybrid assay. Western analysis using an antibody against the GAD moiety in the fusion proteins showed that the mutated and wild-type proteins were all expressed at comparable levels. Mutation of Trp₁₁₇, which is conserved in all classes of E2 and UEV proteins and believed to demarcate the C-terminus

25 of the active site region (Koonin, E. V. & Abagyan, R. A., 1997, *Nat. Genet.* 16,330-331; Hershko, A. & Ciechanover, A., 1998, *Annu. Rev. Biochem.* 67,425-479) eliminated binding completely, indicating that this residue is important in both Tsg101 and Ubc4. Consistent with the hypothesis that residues unique to Tsg101 determined L domain interaction, substitution of 3 of the 4 non-conserved residues tested impaired p6 binding (Fig. 4B).

30 Conservative substitution of Trp for Tyr₁₁₀ reduced p6 interaction to 31% of wild-type. Substitution of the hydrophobic amino acid Val for Tyr₁₁₃ reduced binding to 33% of wild-type. Substitution of Ala for Lys₁₁₈ reduced binding to a lesser extent.

To further test the hypothesis, we examined residues in Tsg101 that are not conserved in E2 enzymes, but align with regions previously shown to determine substrate

35 recognition in the Ubc4 subgroup (Koonin, E. V. & Abagyan, R. A., 1997, *Nat. Genet.*

16,330-331; Ponting, C. P., Cai, Y.-D. & Bork, P., 1997, *J. Mol. Med.* 75,467-469; Oughtred, R., Bedard, N, Vrielink, A. & Wing, S.S., 1998, *J. Biol. Chem.* 273, 18435-18442). Residues 49 and 125 in Ubc4 were found to determine the substrate specificity of structurally homologous, but functionally distinct Ubc4 isoforms (Oughtred, R., Bedard, N, Vrielink, A. & Wing, S.S., 1998, *J. Biol. Chem.* 273, 18435-18442). Residue 49 aligns with Tsg101 Thr₆₇ (Koonin, E. V. & Abagyan, R. A., 1997, *Nat. Genet.* 16,330-331; Sancho, E. Vila, M.R., Sanchez-Pulido, L., Lozano, 3.3., Paciucci, R., Nadal, M., Fox, M., Harvey, C. Bercovich, B., Loukili, N., Ciechanover, A., Lin, S.L., Sanz, F. Estivill, X., Valencia, A. & Thomson, T. M., 1998, *Mol. Cell Biol.* 18, 576-589) or Asn₆₉ (Ponting, C. P., Cai, Y.-D. & Bork, P., 1997, *J. Mol. Med.* 75,467-469). Residue 125 aligns with Ser₁₄₉ (24) or Tyr₁₅₀ (Sancho, E. Vila, M.R., Sanchez-Pulido, L., Lozano, 3.3., Paciucci, R., Nadal, M., Fox, M., Harvey, C. Bercovich, B., Loukili, N., Ciechanover, A., Lin, S.L., Sanz, F. Estivill, X., Valencia, A. & Thomson, T. M., 1998, *Mol. Cell Biol.* 18, 576-589). We therefore substituted Ala for ₆₉Thr-Tyr-Asn₆₉ and ₁₄₉Ser-Tyr₁₅₀ and determined the effect on binding. As shown in FIG. 4C, mutation of ₆₉Thr-Tyr-Asn₆₉ reduced binding to 5% of the wild-type level. Mutation of ₁₄₉Ser-Tyr₁₅₀ had no effect. Western blotting confirmed that the mutants were expressed at wild-type levels. The results are consistent with the conclusion that the interaction of Gag with Tsg101 is based on specific recognition and support the suggestion that the N-terminal E2-like domain of Tsg101 is the minimal determinant of p6 binding.

Discussion

In this example, the interaction of Tsg101 with HIV-1 Pr55^{Gag} *in vitro* and *in vivo* is described. The N-terminal half of Tsg101, which contains the determinant of Gag binding, is homologous to Ub-conjugating E2 enzymes (Koonin, E. V. & Abagyan, R. A., 1997, *Nat. Genet.* 16,330-331; Ponting, C. P., Cai, Y.-D. & Bork, P., 1997, *J. Mol. Med.* 75,467-469; Jentsch, S., Seufert, W., Sommer, T. & Reins, H.A., 1990, *Trends Biochem. Sci.* 15, 195-198). The C-terminal half of Tsg101 has a coiled-coil domain that can interact with a cytoplasmic phosphoprotein, stathmin, implicated in microtubule dynamics (Li, L. & Cohen, S. N., 1996, *Cell* 85, 319-329), and contains a highly conserved sequence that regulates the steady state level of the protein (Smith, A. J., Cho, M. I., Hammariskjold, M. L. & Rekosh, D., 1990, *J. Virol.* 64, 2743-2750). Based on its structural features, Tsg101 has been speculated to be (i) a dominant-negative Ub regulator (Koonin, E. V. & Abagyan, R. A., 1997, *Nat. Genet.* 16,330-331; Ponting, C. P., Cai, Y.-D. & Bork, P., 1997, *J. Mol.*

Med. 75,467-469); (ii), a transcriptional regulator (Sun, Z., Pan, 3., Hope, W. X., Cohen, S. N. & Balk, S. P., 1999, *Cancer* 86,689-96); (iii), a regulator of the cell cycle (Xie, W., Li, L. & Cohen, S. N., 1998, *Proc. Natl. Acad. Sci. USA* 95, 1595-1600; Zhong, Q., Chen, Y., Jones, D. & Lee, W. H., 1998, *Cancer Res.* 58,2699-2702); and (iv), a regulator of
 5 membrane protein trafficking (Babst, M., Odorizzi, G., Estepa, E. J. & Emr, S. D., 2000, *Traffic* 1,242-258; Lemmon, S. K. & Traub, L. M., 2000, *Curr. Opin Cell Biol.* 12,457-466). It is not clear whether these apparently diverse roles reflect independent or related functions of the protein. Furthermore, how participation in these functions may relate to Tsg101's role in the ubiquitination process is unknown.

10 The observation that Tsg101 interacts with HIV-1 Gag in mammalian cells suggests that the interaction is relevant to the viral life cycle. It is noted that the interaction was much more efficient in cytoplasmic extracts than *in vitro*, perhaps suggesting a need for stabilizing cellular factors or a particular Gag assembly state. The fact that the L domain-containing p6 region of the protein is required for binding implicates the interaction
 15 in the late budding function. The results described in this example indicate that deletion of the L domain PTAPP motif prevents the interaction of Gag with Tsg101. This Pro-rich sequence is highly conserved in all lentiviruses except equine infectious anemia virus (Myers, G., Korber, B., Wain-Hobson, S., Smith, R. F. & Pavlakis, G. N., 1995, Los Alamos National Laboratory, Los Alamos, NM). It is duplicated in some isolates of HIV-1,
 20 HIV-2 and simian immunodeficiency virus (SIV). The human T-cell leukemia virus type 1 (HTLV-1) and the Mason-Pfizer monkey virus (MPMV) Gag proteins contain both the lentivirus motif (PT(S)AP) and the (P)PPPY motif, the functionally interchangeable avian retrovirus counterpart (Wills, J. W., Cameron, C. E., Wilson, C. B., Xiang, Y., Bennett, R. P. & Leis, J., 1994, *J. Virol.* 68,6605-6618; Parent, L.J., Bennett, R.P., Craven, R.C., Nelle,
 25 T.D., Krishna, N.K., Bowzard, J.B., Wilson, C.B., Puffer, B.A., Montelaro, R.C. & Wills, J.W., 1995, *J. Virol.* 69, 5455-5460). Studies indicating that the PY and PTAP motifs recruit the Ub machinery and that certain proteasome inhibitors cause alterations in viral particle budding similar to defects resulting from mutations in PTAPP and PY support the possibility that the cell's ubiquitination machinery is linked to viral assembly (Schubert, U.,
 30 Ott, D. E., Chertova, E. N., Welker, R., Tessmer, U., Princiotta, M. F., Bannink, J. R., Krausslich, H. G. & Yewdell, J. W., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13057-13062; Strack, B., Calistri, A., Accola, M. A., Palu, G. & Gottlinger, H. G., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13063-13068; Patnaik, A., Chau, V. & Wills, J. W., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13069-13074). However, it is unclear at this time whether this link reflects a
 35 direct or indirect involvement of Ub: On one hand, mutation of the Lys residues in the p6

domain that are substrates for Ub modification has no apparent effect on virus assembly or release (Ott, D. E., Coren, L. V., Chertova, E. N., Gagliardi, T. D. & Schubert, U., 2000, *Virology* 278, 111-121). On the other hand, covalent linkage of Ub to Gag was shown to rescue the defect in release caused by proteasome inhibitors (Patnaik, A., Chau, V. & Wills, J. W., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13069-13074). Perhaps other Lys residues
 5 serve as Ub substrates when the preferred sites in p6 are not available.

The notion that Tsg101 functions as a dominant-negative Ub regulator is based on the fact that Tsg101 lacks the active site Cys residue that conjugates Ub in active E2 enzymes (Koonin, E. V. & Abagyan, R. A., 1997, *Nat. Genet.* 16,330-331; Ponting, C. P.,
 10 Cai, Y.-D. & Bork, P., 1997, *J. Mol. Med.* 75,467-469). The ubiquitination process requires the sequential action of two or three enzymes (see, e.g., Hershko, A. & Ciechanover, A., 1998, *Annu. Rev. Biochem.* 67, 425-479). E1, an Ub-activating enzyme, binds Ub through a thioester bond, then transfers it to E2. E2 enzymes can function alone or in conjunction with E3 Ub-protein ligases to attach Ub to lysine residues in substrate proteins. Substrates
 15 modified by polyubiquitination are degraded by the proteasome; monoubiquitination serves as a signal for endocytosis (Shili, S. C., Sloper-Mould, K. E. & Hicke, L., 2000, *EMBO J.* 19, 187-198). Although the active site Cys is not conserved in Tsg101, our results suggest that this region functions directly or indirectly in binding of the HIV-1 L domain. It is noteworthy that the residues in the altered Ub-binding site in Tsg101 that contribute to L
 20 domain PTAPP binding are Tyr and Trp residues flanked by positively charged Lys residues (Fig. 4B). Aromatic amino acids flanked by charged residues are critical binding determinants for protein binding motifs like SH3 and WW domains. SH3 domains bind Pro-rich sequences having the consensus PXXP, like PTAPP; WW domains interact with PPXY motifs (Kay, B. K., Williamson, M. P. & Sudol, M., 2000, *FASEBJ.* 14, 231-241;
 25 Gamier, L., Wills, J. W., Verderame, M. F. & Sudol, M., 1996, *Nature* 381, 744- 745). Moreover, as the amino acids surrounding these aromatic residues in Tsg101 are conserved in active E2 enzymes, Tsg101 may maintain an E2-like ability to present Gagp6 to interacting E3 enzymes, as suggested for E2-Ub-E3 interacting complexes (Laney, J. D. & Hochstrasser, M., 1999, *Cell* 97, 427-430; Nuber, U. & Schefflier, M., 1999, *J. Biol. Chem.*
 30 274, 7576-7582). If so, the Tsg101-Gag-complex may associate with an active E3 enzyme to facilitate an event related to L domain function. Furthermore, Tsg101 has been shown to function in membrane protein transport (Babst, M., Odorizzi, G., Estepa, E. J. & Emr, S. D., 2000, *Traffic* 1, 242-258; Lemmon, S. K. & Traub, L. M., 2000, *Curr. Opin Cell Biol.* 12,457-466). Its involvement in regulation of vesicles that are required for recycling of
 35 membrane-associated proteins (Babst, M., Odorizzi, G., Estepa, E. J. & Emr, S. D., 2000,

Traffic 1, 242-258; Bishop N and Woodman P., 2001, *J Biol Chem.* 276:11735-42) may permit it to play a role in Gag trafficking to the site of particle maturation and release.

The finding that HIV-1 Gag binds through the E2-like domain in Tsg101 suggests several hypotheses. The interaction of Gag with Tsg101 may be adventitious, based on
 5 Tsg101's similarity to active E2 enzymes. Alternatively, if Ub is required for assembly as suggested (Schubert, U., Ott, D. E., Chertova, E. N., Welker, R., Tessmer, U., Princiotta, M. F., Bennink, J. R., Krausslich, H. G. & Yewdell, J. W., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13057-13062; Strack, B., Calistri, A., Accola, M. A., Palu, G. & Gottlinger, H. G., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13063-13068; Patnaik, A., Chau, V. & Wills, 3. W.,
 10 2000, *Proc. Natl. Acad. Sci. USA* 97, 13069-13074), Tsg101 may function as a cellular defense mechanism that prevents Gag interaction with active E2 enzymes. It is also possible that Tsg101 is recruited by the virus as a chaperone to block Gag polyubiquitination and subsequent degradation by the proteasome. This idea is supported by the fact that cyclin-specific E2 enzymes with Ser substituted for the active Cys are, in
 15 fact, dominant negative inhibitors of cyclin destruction (Townesley, F. M., Aristarkhov, A., Beck, S., Hershko, A. & Ruderman, J. V., 1997, *Proc. Natl. Acad. Sci. USA* 94,2362-2367). Finally, Tsg101 may function like the yeast UEV Mms2 protein, which alters the function of interacting E3 proteins (Hofmann, R. M. & Pickart, C. M., 1999, *Cell* 96, 645-653). Interestingly, the L domain of the Ebola virus matrix protein interacts with Nedd4, an E3
 20 Ub protein ligase (Harty, R. N., Brown, M. E., Wang, G., Huibregtse, J. & Hayes, F. P., 2000, *Proc. Natl. Acad. Sci. USA* 97, 13871-13876). The apparent conservation of L domain interaction with cellular proteins that affect Ub modification suggests that the involvement of the Ub machinery is a highly conserved event in virus assembly and particle release both within and outside the retrovirus family.

25

6.2. EXAMPLE 2

Example 1 demonstrates that the PTAPP motif or L domain of human immunodeficiency virus type 1 (HIV-1), which is located in the C-terminal p6 region of the Gag precursor polypeptide, was found to bind to the protein product of the *tsg101* gene.
 30 Sequence analysis has suggested, and experimental evidence has shown, that Tsg101 can function in both the modulation of transcription and the inhibition of ubiquitination and protein decay. The latter effects are mediated by an N-terminal region that contains a ubiquitin (Ub) conjugase (E2)-like domain, but lacks an active site Cys residue crucial to Ub conjugation. This domain of Tsg 101 is the minimal binding region required for its
 35 interaction with HIV-1 Gag. The involvement of Tsg101 in endosomal trafficking, together

with its ability to interact with HIV-1 Gag *in vitro* and in mammalian cells as shown in example 1, and the finding that the L domain in the p6 region of Gag is specifically required for binding, have led to studies in this example to investigate the effects of Tsg101 on the late budding function responsible for mature virus release from the cell surface.

- 5 In this example, it is shown that the release of mature HIV-1 particles is reduced in murine SL6 cells, which are deficient in Tsg101 (Li, L. & Cohen, S. N., 1996, *Cell* 85, 319-329), as well as in primate COS-1 cells that overproduce the wild-type Tsg101 protein. It has also been shown that SL6 cells are defective in sorting of multiple surface-bound proteins (Babst, M., Odorizzi, G., Estepa, E. J. & Emr, S. D., 2000, *Traffic* 1, 242-258).
- 10 Mutation of Tsg101 in a region necessary for regulation of Tsg101 protein decay, but not for Gag binding (see Example 1), partially reversed the inhibitory effects of Tsg101 overexpression on virus release. The effects of Tsg101 overproduction required the presence in Gag of sequences that interact with Tsg101. The results in this example provide evidence that the Tsg101 protein specifically regulates the transport and maturation of HIV-1
- 15 particles through its interaction with the L domain of Gag, implicating Tsg101 function in productive virus assembly.

- The p6 region in Pr55^{Gag}, the structural precursor polyprotein encoded by human immunodeficiency virus type I (HIV-1), directs release of mature virus-like particles from the plasma membrane of *gag*-transfected cells. Example 1 demonstrates that Gag^{p6} binds to
- 20 Tsg101, a protein that affects trafficking of membrane-associated proteins. In this example, it is demonstrated that Tsg101 deficiency induced by functional inactivation of Tsg101 mRNA by an antisense transcript prevented Gag protein maturation in a cellular fraction enriched in plasma membranes and reduced virus particle release, suggesting that Tsg101 is required for normal Gag trafficking and processing. Paradoxically, overexpression of
- 25 Tsg101 also inhibited the release of virus particles but did not affect exit of Gag Δ p6, which does not bind Tsg101. Inhibition was reduced by mutation of the Tsg101 C-terminal steadiness box (SB) domain, which is required for proper autoregulation of Tsg 101 decay. However, while the SB mutation decreased the ability of overexpressed Tsg101 to interfere with virus particle release, maturation of the particle-associated Gag precursor protein was
- 30 defective. The results in this example suggest that Tsg101 has a role in both the maturation and release of HIV particles, potentially providing a novel target for interfering with HIV-1 production.

Materials And Methods

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Plasmids. *pgp-RRE-r* (Smith, A. J., Cho, M. I., Hammarskjold, M. L. & Rekosh, D., 1990, *J. Virol.* **64**, 2743-2750), encoding wild-type *gag*, *pol*, and *vif* was used as a template to synthesize Pr55 Gag Δ p6 by engineering three point mutations in the first codon of p6 by PCR, converting it to a stop (ochre) codon. The 5' primer

5 (GGAAGATCTGGCCTTCCTACAAGGGAAGGCCAGGGAATT, SEQ ID NO:37) anneals to nt 1637 to 1687 of BH10 *gag* (Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K. & et al., 1985, *Nature* **313**, 277-284) and includes a unique BglII site. The 3' primer (CCATGTATTGATAGATAACTATGTCTG, SEQ ID NO:38) anneals to nt 2655 to 2682

10 in the *pol* gene downstream of a unique EcoRV site. The *pgp-RRE-r* plasmid and the fragment produced from PCR were cut with BglII and EcoRV and the desired fragments were ligated. The mutation was confirmed by sequencing with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA). pIND-hTsg101-FLAG (wild-type human Tsg101) and pIND-SD5-59-FLAG, in which Gly

15 replaces amino acids 348-351, are described (Feng, G.H., Lih, C.-J., & Cohen, S.N., 2000, *Cancer Research* **60**, 1736-1741).

Cell culture, transfection, and preparation of cytoplasmic extracts. COS-1 cells were cultured in DMEM supplemented with fetal bovine serum and antibiotics to 60%

20 confluency at 37°C. The cells were transfected with wild-type or mutated pIND-hTsg101-FLAG, pVgRXR, pCMV-*rev*, and wild-type or mutated *pgp-RRE-r* in various combinations using the FuGene 6 reagent (Roche). Rev is a HIV-1-encoded protein required for expression of *gag* and *pol* (Frankel, A. D. & Young, J. A., 1998, *Annu. Rev. Biochem.* **67**, 1-25). PVgRXR encodes both monomers of a heterodimeric ecdysone-inducible receptor that,

25 together with the hormone, form a transcriptional activator complex that binds the promoter driving *tsg101* expression. Treating the transfected cells with the ecdysone analogue, pronasterone, for 24 hr at 24 hr after transfection induced expression of Tsg101. At 48 hr post-transfection, the cells were harvested by scraping into the media and collected by centrifugation. The pelleted cells were washed three times with cold PBS, swollen in 1 ml

30 of cold hypotonic buffer (10 mM Tris, pH 7.4, 1 mM MgCl₂) containing protease inhibitors (15 min, 4°C) and disrupted with 35 strokes of a Dounce homogenizer (type B pestle). The lysate was spun 10 min, 1,000 x g at 4°C to remove unbroken cells, nuclei, and mitochondria. NIH 3T3 and SL6 cells were maintained at 37°C in DMEM supplemented with antibiotics and fetal bovine serum. SL6 cells are 3T3 derivatives that have undergone

35 functional inactivation of the *tsg101* gene by antisense RNA using a previously described

random homozygous knockout (RHKO) procedure (Li, L. & Cohen, S. N., 1996, *Cell* 85, 319-329). These cells were transfected with pHIV-AP Δ env (Sutton, R.E., Wu, H.T., Rigg, R., Bohnlein, E. & Brown, P.O., 1998, *J. Virol.* 72, 5781-5788) and, where indicated in the text, with pME VSV G (Sutton, R.E., Wu, H.T., Rigg, R., Bohnlein, E. & Brown, P.O., 5 1998, *J. Virol.* 72, 5781-5788) using the FuGene 6 reagent. Fractionation into total cytoplasm (S1), nuclei/Golgi-enriched (P1), soluble (S100) and plasma membrane-enriched (P100) fractions was performed as described (L. Alland, S. M. Peseckis, R. E. Atherton, L. Berthiaume & Resh, M.D., 1994, *J. Biol. Chem.* 269, 16701-16705). Virus particles were isolated from culture supernatant passed through a 0.45 μ m filter by ultracentrifugation 10 through a cushion of 20% sucrose at 30,000 rpm for 80 min at 4°C (Beckman SW41 rotor). The pelleted viral particles were resuspended in 1 ml of phosphate buffered saline by gentle shaking at 4°C. Infectivity of VSV G pseudotyped virus preparations was assessed using the MAGI assay (Vodicka, M.A., Goh, W.C., Wu, L.I., Rogel, M.E., Bartz, S.R., Schweickart, V.L., Raport, C., & Emerman, M., 1997, *Virol.* 233, 193-198).

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Immunoprecipitation assay. Protein A agarose beads (Pierce), pre-washed with non-denaturing hypotonic (10 mM Tris, pH 7.4; 1 mM MgCl₂) buffer containing protease inhibitors (Roche), were incubated with the appropriate antibody, added to cytosolic extracts and rotated at 4°C for 60 min. Immunoprecipitates were washed in hypotonic 20 buffer, boiled in SDS sample buffer with 5% mercaptoethanol, resolved by SDS-PAGE and analyzed by Western blotting with the antibodies indicated in the text.

Protein detection. Proteins were separated by electrophoresis through 12.5% SDS-polyacrylamide gels. Following electrophoresis, the gels were transferred to nitrocellulose 25 and analyzed by Western blotting. The following antibodies were used, as specified in the text: anti-Tsg101 mouse monoclonal antibody (Santa Cruz, Inc); anti-Tsg101 rabbit polyclonal antipeptide antibody raised against amino acids 383-391; anti-FLAG mouse monoclonal (obtained from Sigma); anti-CA rabbit polyclonal antibody raised against a native form of the CA protein (Ehrlich, L.S., Krausslich, H.-G., Wimmer, E. & Carter, C.A., 30 1990, *AIDS & Hum. Retro.* 6, 1169-1175.); anti-CA mouse monoclonal antibody (NEN-DuPont); anti-p6 rat monoclonal antibody (ABI). Proteins were visualized by chemiluminescence using Lumi-Light reagents (Roche).

Results

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Effect of Tsg101 deficiency on virus production. Example 1 has demonstrated that Tsg101 associates specifically with the L domain in HIV-1 Pr55 Gag^{pb}, a region that directs exocytosis of mature viral particles from infected cells (Göttlinger, H. G., Dorfman, T., Sodroski, J. G. & Haseltine, W. A., 1991, *Proc. Natl. Acad. Sci. USA* **88**, 3195-3199; Huang, M., Orenstein, J. M., Martin, M. A. & Freed, E. O., 1995, *J. Virol.* **69**, 6810-6818). To investigate the role of this interaction in viral production, the effect of modulating *tsg101* expression on viral particle release was determined. As an initial approach, virus production was examined in a NIH 3T3-derived cell line (SL6) in which Tsg101 expression has been reduced to ~30% of normal levels using an antisense strategy (Li, L. & Cohen, S. N., 1996, *Cell* **85**, 319-329).

For these experiments, Gag and all of the other viral proteins except Nef and Env were expressed from pHIV-AP Δ env (Sutton, R.E., Wu, H.T., Rigg, R., Bohnlein, E. & Brown, P.O., 1998, *J. Virol.* **72**, 5781-5788) and the virus particles released into the media were assessed by Western blotting. pHIV-AP Δ env contains the human placental alkaline phosphatase gene in place of the *nef* gene and the *env* gene has been deleted. Growth conditions were identified to obtain cell cultures matched for extent of confluency as measured by total protein staining and Western blot analysis of specific subcellular compartment markers. In control experiments, it is ascertained that SL6 was transfected as efficiently as 3T3 using the Fugene6 reagent. Cytoplasmic (S1) extracts prepared from matched cultures of transfected parental and mutant cells and exhibiting total protein values within $\pm 0.01 \mu\text{g}$ of each other showed identical Coomassie blue stained protein patterns (Fig. 5A, lanes 1, 2) and comparable expression of Na⁺K⁺ATPase and actin (lanes 3,4). In contrast, as previously reported, SL6 cells accumulated Tsg101 at ~30% the level of the parental cells, as determined by comparative semi-quantitative Western analysis using an anti-Tsg101 monoclonal.

Analysis of cell-associated Gag protein in 3T3 and SL6 cells. To determine the effect of Tsg101 deficiency on the intracellular accumulation of Gag, the transfected cells were subjected to differential centrifugation and the amount of viral protein was assessed in the S1 lysate and in subcellular fractions. The S1 lysate prepared from transfected 3T3 cells, but not mock-treated cells, contained mature CA protein (FIG. 5C, lanes 1, 2), which accumulated in the plasma membrane-enriched P100 fraction (*inset* to FIG. 5C). This fraction was identified by localization of Na⁺K⁺ATPase, an integral plasma membrane protein (see, e.g., L. Alland, S. M. Peseckis, R. E. Atherton, L. Berthiaume & Resh, M.D., 1994, *J. Biol. Chem.* **269**, 16701-16705). In contrast, no mature CA was detected in the S1

fractions prepared from mock-treated or transfected SL6 cells (FIG. 5C, lanes 3, 4). Further analysis of the S1 and P1 fractions from the SL6 cells using an antibody that recognizes the Gag precursor confirmed the absence of virus-specific protein in the S1 fractions prepared from these untreated or transfected SL6 cells (FIG. 5D, lanes 1,2). In contrast, the antibody
5 revealed the full-length Gag precursor in the P1 fraction (lane 4), which is enriched in filamentous actin-, Golgi complex-, and nuclear proteins (see, e.g., L. Alland, S. M. Peseckis, R. E. Atherton, L. Berthiaume & Resh, M.D., 1994, *J. Biol. Chem.* **269**, 16701-16705). These results suggest that the block to virus release in SL6 cells resulted from defective transport of the Gag precursor to the plasma membrane where it normally
10 assembles, and consequently implicate Tsg101 in this transport function.

Effect of Tsg101 overproduction on release of HIV-1 particles. The steady-state level of Tsg101 protein is maintained within a narrow range during cell growth by a post-translational mechanism involving protein degradation (Feng, G.H., Lih, C.-J., & Cohen,
15 S.N., 2000, *Cancer Research* **60**, 1736-1741). Overexpression of a FLAG-tagged Tsg101 protein leads to accelerated turnover of both the endogenous and FLAG-tagged protein (Feng, G.H., Lih, C.-J., & Cohen, S.N., 2000, *Cancer Research* **60**, 1736-1741). Use of a pIND-h-Tsg101-FLAG construct (Feng, G.H., Lih, C.-J., & Cohen, S.N., 2000, *Cancer Research* **60**, 1736-1741) enables inducible expression of FLAG-tagged Tsg101 under
20 control of a modified promoter containing *Drosophila* ecdysone-responsive DNA elements. In preliminary experiments using the ecdysone analog, pronasterone inducer (hereinafter "pa"), the conditions that allowed to overproduce FLAG-tagged Tsg101 without drastic perturbation of the steady-state level of endogenous protein was identified. As shown in
25 Figure 6A, Western analysis using anti-Tsg101 antibody detected an endogenous protein band migrating at the expected ~46 kDa position and increasing amounts of a protein that migrated more slowly. Re-probing the blot with antibody against the eight residue FLAG sequence confirmed that the more slowly migrating protein was FLAG-tagged Tsg101 derived from expression of the induced plasmid encoding the protein.

As previously demonstrated, plasmid *pgp-RRE-r*, when co-expressed in COS cells
30 with the HIV-1 regulatory protein, Rev, allows assembly and release of virus-like particles that are indistinguishable from mature HIV-1 (Smith, A. J., Cho, M. I., Hammariskjold, M. L. & Rekosh, D., 1990, *J. Virol.* **64**, 2743-2750, Ebbets-Reed, D., Scarlata, S. & Carter, C.A. *Biochem.* **35**, 14268-14275). To ensure the specificity of any Tsg101-related effects we observed, the HIV-1 Gag Δ p6 mutant was examined in parallel. The mutated Gag protein (*i*)
35 lacks the p6 domain that binds Tsg101; (*ii*) assembles particles that fail to complete

proteolytic maturation; and (iii) is released inefficiently (see, Example 1; Göttinger, H. G., Dorfman, T., Sodroski, J. G. & Haseltine, W. A., 1991, *Proc. Natl. Acad. Sci. USA* 88, 3195-3199; Huang, M., Orenstein, J. M., Martin, M. A. & Freed, E. O., 1995, *J. Virol.* 69, 6810-6818). It was found that particles containing Gag Δ p6 are released at 50% wild-type efficiency, as measured by comparing accumulation in the cytoplasm and media. The effect of increasing amounts of Tsg101 on release of virus particles was determined by adding the pa inducer at 0, 1, and 5 μ M to cells transfected with a constant amount of pIND-h-Tsg101-FLAG DNA, *rev*, *pol* and *gag* or *gag* Δ 6 and examining the culture media for virus-like particles. As expected, no virus-specific protein was released from cells expressing only *rev* (Figure 6B, lane 1). Viral particles released from cells treated with 0 μ M pa contained the expected band of mature CA protein (lane 2). The virus released from cells treated with 1 or 5 μ M pa exhibited the same mature CA composition, but the amount was reduced to ~60% and ~30%, respectively, of the control (0 μ M pa) level (lanes 3 and 4, respectively). Addition of 5 μ M pa in the absence of pIND-h-Tsg101-FLAG had no inhibitory effect. Release of immature particles containing bands identified as the truncated Gag Δ p6 precursor protein and MA-CA was not significantly affected by Tsg101 overexpression and remained essentially unchanged (lanes 5-7). The results indicate that increased intracellular levels of Tsg101 interfered with release of wild-type Pr55^{Gag} particles and that this inhibition required the presence of an intact p6 domain in Gag.

Effect of mutation of the Tsg101 SB domain. Taken together, the results above suggested that *both* Tsg101 deficiency and overproduction could impair mature virus particle release. However, it was also possible that the over-expressed FLAG-tagged Tsg101 protein was acting as a dominant-negative inhibitor that interfered with normal trafficking. As described above, the C-terminally located SB domain regulates the steady-state level of Tsg101 post-translationally by affecting degradation of the Tsg101 protein (Feng, G.H., Lih, C.-J., & Cohen, S.N., 2000, *Cancer Research* 60, 1736-1741), and recent evidence has implicated the 26S proteasome in this process (Li, L., Liao, J., Ruland, J., Mak, T.W., & Cohen, S.N., 2001, *Proc. Natl. Acad. Sci. USA* 98, 1619-1624). It is hypothesized that the observed reduction in viral particle release upon Tsg101 over-expression may result from adventitious degradation of Tsg101-associated Gag protein due to events associated with autoregulation. This notion predicts that over-expression of Tsg101 proteins mutated in the SB region and deficient in Tsg101 autoregulation should not lead to inhibition of virus release. The Tsg101 mutant SD5-59 protein contains a highly localized mutation in which a glycine residue replaces 4 aa (residues 348-351) in the SB domain and consequently is

partially defective in its ability to down-regulate the Tag101 protein (Feng, G.H., Lih, C.-J., & Cohen, S.N., 2000, *Cancer Research* 60, 1736-1741). Western blot analysis confirmed that this protein accumulated following addition of the pa inducer or in response to transfection of increasing amounts of the mutated Tsg101-FLAG DNA (Fig. 7A).

5 The effect of increasing expression of SD5-59 on release of virus particles was determined by adding pa at 0, 1, and 5 μ M to COS cells transfected with the mutant and *rev*, *pol* and *gag* or *gag* Δ 6 and examining the culture media for virus-like particles. As expected, no virus-specific protein was released from cells expressing only *rev* (FIG. 7B, lane 1). The particles released from cells treated with 0 μ M pa exhibited the CA composition of the
 10 mature virus particle (lane 2), consistent with the results described above (Figure 6B, lane 2). Consistent with the hypothesis that events related to Tsg101 autoregulation interfere with virus production, expression of increasing amounts of the SD5-59 mutant protein-- which is inefficient in regulation--permitted release of virus particles (lanes 3,4). Surprisingly, however, expression of the mutated Tsg101 protein also resulted in
 15 accumulation of particle-associated uncleaved Gag precursor. In contrast, particles assembled by Gag Δ p6 were identical to those detected when wild-type *tsg101* was over-expressed (lanes 5-7). The observation that overexpression of the Tsg101 mutant resulted in release of particles containing unprocessed Gag suggests that the mutation, while allowing normal virus release, disengaged the usual coupling of virus release and Gag
 20 processing.

Deletion of aa348-351 is not expected to affect Tsg101 interaction with Gag because the binding site for Gag in Tsg101 resides in the N-terminal Ub E2 variant (UEV) domain. C-terminally truncated proteins lacking residues 348-351 bind Gag^{p6} as efficiently as the intact protein in a 2-hybrid assay. As shown in FIG. 8, Western analysis using anti-p6
 25 antibody indicated that the Gag^{p6} precursor and the NC-p6 cleavage product accumulated in cytoplasmic extracts prepared from induced cells transfected with *gag* and 2 μ g of either the wild-type or mutated *tsg101* DNA (FIG. 8A, lanes 3,4). Anti-Tsg101 antibody immunoprecipitated the viral proteins in the 2 extracts to a comparable extent (FIG. 8B, lanes 3,4), indicating that expression of the exogenous proteins did not change the amount
 30 of Gag bound to Tsg101. Re-probing the blot with antibodies against the FLAG tag (FIG. 8C) or the full-length Tsg101 protein (FIG. 8D) confirmed the presence of the exogenous (FLAG-tagged) and native Tsg101 proteins in the immune precipitate (lanes 1-4). These results indicate that lack of effect of the SD5-59 mutation on Gag release were not due to abrogation of the Tsg101-Gag interaction.

35 To further test the supposition that the inhibition of virus particle release by

overexpression of the wild-type Tsg101 protein results from events associated with Tsg101 autoregulation, the steady-state level of viral protein under conditions where the greatest effect on release was detected was assessed. As shown in Fig. 9A, control cells transfected with *gag*, *pol*, and *rev* but no exogenous *tsg101* accumulated high levels of viral protein in both the S1 and P1 fractions, as revealed by Western analysis with anti-CA antibody (lanes 2, 4). In contrast, cells transfected with *gag*, *pol*, *rev* and wild-type *tsg101-FLAG* DNA under conditions that yielded the greatest inhibition (*c.f.* Figure 6B, lane 4) contained no detectable viral protein in the S1 or P1 fractions, suggesting that extensive degradation of viral protein had occurred (Fig. 9B, lanes 1, 3). This effect required the p6 domain of Gag, as Gag Δ p6 was detected in both S1 and P1 fractions (lanes 2,4). Under the same conditions, cells expressing the SD5-59 mutant instead of the wild-type Tsg101 protein accumulated detectable levels of viral protein in the S1 fraction (panel C, lane 1), although no protein was found in the P1 compartment (lane 3). In contrast, Gag Δ p6 accumulated at higher levels in both fractions (lanes 2,4). The results support the hypothesis that the Tsg101 autoregulatory mechanism affects the steady-state level of intracellular Gag.

Discussion

In this example, it is shown that perturbation of the steady-state level of Tsg101 by under- or over- expression of the protein negatively affected release of mature HIV-1 virus-like particles from murine and primate cells. Mutation of the Tsg101 SB region, which has been implicated in Tsg101-mediated post-translational autoregulation of its steady-state protein level reduced the ability of the over expressed Tsg101 protein to block virus particle release, as did mutation of the p6 domain. These results link the production of HIV-1 virus particles directly to a cellular protein (*i.e.*, Tsg101) that binds the Gag L domain, which controls egress of mature virus, and provide strong evidence that the Gag-Tsg101 interaction plays a specific and significant role in HIV-1 propagation. The fact that modulation of Tsg101 expression in both murine (SL6) and primate (COS) cells affected virus release indicates that Tsg101 is an important cofactor in HIV-1 virus assembly in diverse intracellular environments.

The observation that over expression of a Tsg101 protein mutated in the SB domain resulted in release of virus particles containing Gag that was not proteolytically processed indicates that Tsg101 is involved in the Gag protein maturation associated with virus particle release. The effect of Tsg101 on release of mature virions may be a consequence of Tsg101's proposed regulation of the endocytic pathway (Lemmon, S. K. & Traub, L. M.,

2000, *Curr. Opin. Cell Biol.* **12**, 457-466). Interestingly, the mutation in SD5-59 that partially inactivates the autoregulatory mechanism lies in a region that is required for interaction with another endocytic sorting protein, Vps28 (Bishop N and Woodman P., 2001, *J Biol Chem.* **276**:11735-42), which is included with Tsg101 in a complex that
5 functions in trafficking of proteins between the plasma membrane and endocytic vesicles (Babst, M., Odorizzi, G., Estepa, E. J. & Emr, S. D., 2000, *Traffic* **1**, 242-258).

Potentially, the SD5-59 mutation may have disengaged Tsg101 from the endocytic sorting machinery, causing Gag to bypass a processing site and be released in an uncleaved form. This possibility is supported by the observation that the Gag Δ p6 mutant, which does
10 not bind Tsg101 and would therefore not engage the machinery, is also released in an unprocessed form.

In this regard, it is noteworthy that Tsg101-deficient SL6 cells exhibited defects in sorting and proteolytic maturation of the lysosomal hydrolase cathepsin D that are presumed to be due to failure to deliver the enzyme to the processing site in the lysosome (Babst, M.,
15 Odorizzi, G., Estepa, E. J. & Emr, S. D., 2000, *Traffic* **1**, 242-258). The results in this example also associate Tsg101 deficiency with defective transport of Gag precursors to their normal plasma membrane maturation site. Impaired trafficking of Gag in SL6 cells may limit the pool of Gag precursors available at the membrane budding site, resulting in reduction in the amount of released particles, as we observed. As Gag lacking p6 associates
20 with the membrane efficiently (see, e.g., Göttlinger, H. G., Dorfman, T., Sodroski, J. G. & Haseltine, W. A., 1991, *Proc. Natl. Acad. Sci. USA* **88**, 3195-3199) even though it is released less effectively (Göttlinger, H. G., Dorfman, T., Sodroski, J. G. & Haseltine, W. A., 1991, *Proc. Natl. Acad. Sci. USA* **88**, 3195-3199), transport to a *specific* processing site or membrane compartment, rather than membrane localization *per se*, may be the Gag-
25 related event that requires normal levels of the native Tsg101 protein. Alternatively, as proteolytic processing requires the viral protease-containing Gag-Pol precursor (Swanstrom, R. & Wills, J. W., 1997, in *Retroviruses*, eds. Coffin, J. M., Hughes, S. H. & Varmus, H. E., Cold Spring Harbor Laboratory Press, New York, pp. 263-334), the change in efficiency of proteolytic processing mediated by the SD5-59 mutant may reflect differential effects of
30 Tsg101 on Gag^{p6} but not Gag-Pol (which lacks p6). In any case, the fact that a mutation that may have disengaged Tsg101 from the endocytic machinery reduced the efficiency of proteolytic processing in the particles assembled in COS-1 cells links proteolytic maturation and release of HIV-1 Gag to proper functioning of proteins in the endocytic machinery. Perhaps the putative processing compartment or differential trafficking event whose
35 existence is suggested by our results indirectly involves the proteasome, as proteasome

inhibitors were found to prevent cell-mediated degradation of Tsg101 (Li, L., Liao, J., Ruland, J., Mak, T.W., & Cohen, S.N., 2001, *Proc. Natl. Acad. Sci. USA* **98**, 1619-1624) and viral protease-mediated processing of Gag (Schubert, U., Ott, D. E., Chertova, E. N., Welker, R., Tessmer, U., Princiotta, M. F., Bennink, J. R., Krausslich, H. G. & Yewdell, J. W., 2000, *Proc. Natl. Acad. Sci. USA* **97**, 13057-13062.).

Regarding Tsg101 control of virus release, these results suggest that the Tsg101 steady-state level and the efficiency of mature virus release are tightly coupled. Tsg101 deficiency and overproduction resulted in similar outcomes, *i.e.*, reduced Gag accumulation in the plasma membrane-enriched S1 fraction. This example demonstrates that Tsg101 over-expression correlated with apparent degradation of Gag and that the lessened inhibitory effect of the SD5-59 mutant protein was related to reduced effects on Gag imposed by autoregulation of Tsg101. Interestingly, under the conditions of these experiments, Gag was much more affected by over-expression of an exogenously-derived *tsg101* gene than was the endogenous Tsg101 protein. This observation suggests that the form of Gag that binds Tsg101 is a multimer and that Tsg101 interaction with a subset of molecules in the multimer was sufficient to induce the observed inhibitory effects on Gag accumulation and release. This seems consistent with previous observations that efficient particle release does not require that every Gag molecule possess an L domain (Wills, J. W., Cameron, C. E., Wilson, C. B., Xiang, Y., Bennett, R. P. & Leis, J., 1994, *J. Virol.* **68**, 6605-6618), the binding site of Tsg101 in Gag as shown in Example 1. The observation also may suggest that only a subpopulation of Tsg101 molecules can form productive complexes with Gag, as the inhibition of mature virus production was aggravated by greater accumulation of the wild-type FLAG-tagged Tsg101 protein. Recent evidence indicates that Tsg101 can interact with Ub ligases Mdm2 (Li, L., Liao, J., Ruland, J., Mak, T.W., & Cohen, S.N., 2001, *Proc. Natl. Acad. Sci. USA* **98**, 1619-1624) and Nedd4. These enzymes monoubiquitinate substrates destined for transport by the endocytic machinery (Shih, S. C., Sloper-Mould, K. E. & Hicke, L., 2000, *EMBO. J.* **19**, 187-198) and polyubiquitinate cargo intended for proteasomal degradation (Hershko, A. & Ciechanover, A., 1998, *Annu. Rev. Biochem.* **67**, 425-479), possibly including Tsg101 itself when it is overproduced. The notion that Tsg101 functions in concert with Ub ligases is supported by studies that implicate the Ub machinery in L domain function and viral exocytosis (Schubert, U., Ott, D. E., Chertova, E. N., Welker, R., Tessmer, U., Princiotta, M. F., Bennink, J. R., Krausslich, H. G. & Yewdell, J. W., 2000, *Proc. Natl. Acad. Sci. USA* **97**, 13057-13062, Strack, B., Calistri, A., Accola, M. A., Palu, G. & Gottlinger, H. G., 2000, *Proc. Natl. Acad. Sci. USA* **97**, 13063-13068; Patnaik, A., Chau, V. & Wills, J. W., 2000, *Proc. Natl. Acad. Sci. USA*

97, 13069-13074; Ott, D. E., Coren, L. V., Chertova, E. N., Gagliardi, T. D. & Schubert, U., 2000, *Virology* 278, 111-121; Vogt, V. M., 2000, *Proc. Natl. Acad. Sci. USA* 97, 12945-12947). Tsg101 interaction with Ub ligases may bring these enzymes into proximity with assembling Gag particles. Gag may require tight Tsg101-mediated control to promote
 5 transport vs. degradation.

Precisely how Tsg101 might mediate trafficking of Gag is unclear. Possibly, Tsg101 controls trafficking of a membrane-associated cellular protein that regulates exocytosis. It is also noteworthy that the invagination event associated with formation of the sorting multi vesicular body (MVB) that Tsg101 is believed to regulate (Lemmon, S. K.
 10 & Traub, L. M., 2000, *Curr. Opin. Cell Biol.* 12, 457-466) is the only example in the cell, other than virus budding, where vesicle formation is directed *away* from the cytoplasm. Thus Gag, through its interaction with Tsg101, may become linked to other vps homologues that determine formation of the sorting MVB and may cause the machinery to direct invagination out of the cell of vesicles containing virus particles. If this strategy is indeed
 15 unique to the virus, the requirement for the interaction may provide a potential target for interfering with HIV propagation.

7. REFERENCES CITED

All references cited herein are incorporated herein by reference in their entirety and
 20 for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific
 25 embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims along with the full scope of equivalents to which such claims are entitled.

30 SEQ. ID. NO. 1:

```

MAVSESQLKK MMSKYKYRDL TVRQTVNVIA MYKDLKPVLD SYVFNDGSSR ELVNLGTGIP 60
VRYRGNINYI PICLWLLDTY PYNPPICFVK PTSSMTIKTG KHDANGKIY LPYLHDWKHP 120
RSELLELIQI MIVIFGEEPP VFSRPTVSAS YPPYTATGPP NTSYMPGMPS GISAYPSGYP 180
PNPSGYPGCP YPPAGYPAT TSSQYPSQPP VTTVGPSRDG TISEDTRAS LISAVSDKLR 240
WRMKEEMDGA QAEINALKRT EEDLKKGHQK LEEMVTRLDQ EVAEVDKNIE LLKKKDEELS 300
SALEKMEQNS ENNDIDEVII PTAPLYKQIL NLYAEENAIE DTIFYLGEAL RRGVIDLDVF 360
35 LKHVRLLSRK QFQLRALMQK ARKTAGLSDL Y 391
  
```

SEQ. ID. NO. 2

MAVSESQLKK MVSKEYKYRDL TVRETVNVIT LYKDLKPVLD SYVFNOGSSR ELMNLTGTIP 60
VPYRGNTYNI PICLWLLDTY PYNPPICFVK PTSSMTIKTG KHDANGKIY LPYLHEWKHP 120
QSDLLGLIQV MIVVFGDEPP VFSRPISASY PPYQATGPPN TSYPGMPGG ISPYPSGYPP 180
NPSGYPGCPY PPGGPYPATT SSQYPSQPPV TTVGPSRDGT ISED TIRASL ISAVSDKLRW 240
5 RMKEEMDRAQ AELNALKRTE EDLKKGHQKL EEMVTRLDQE VAEVDKNIEL LKKKDEELSS 300
ALEKMEHQSE NNDIDEVIIP TAPLYKQILN LYAEENAIED TIFYLGEALR RGVIDLDVFL 360
KHVRLLSRKQ FQLRALMQKA RKTAGLSPLY 390

SEQ. ID. NO:3

PTAPP 5

10 SEQ ID NO:4

ALQSRPEPTA PPEES 15

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WHAT IS CLAIMED IS:

1. A method for identifying a peptide or fragment derived from a mammalian Tsg101 protein, wherein the peptide or fragment is effective in reducing HIV particle production, said method comprising:

5

(a) introducing an expression construct into a mammalian cell or cells, wherein the expression constructs comprises a portion of the coding sequence for a mammalian *tsg101* gene;

10

(b) introducing one or more expression constructs into the mammalian cell or cells, wherein the one or more expression constructs comprises the HIV *gag*, *pol*, and *rev* coding sequences;

15

(c) incubating the transfected mammalian cell or cells in a suitable media for a sufficient time and at a temperature of about 37C to obtain a mammalian cell culture comprising mammalian cells which express a gene product encoded by the portion of a coding sequence for a mammalian *tsg101* gene and gene products encoded by the HIV *gag*, *pol* and *rev* genes;

20

(d) measuring the level of particle associated p24 in the mammalian cell culture; and

25

(e) correlating a reduced level of particle-associated p24, when compared to a control mammalian cell culture which has not been transfected with the expression construct comprising a portion of the coding sequence for a mammalian *tsg101* gene, with the identification of a peptide or fragment effective in reducing HIV particle production.

2. A method for identifying a peptide or fragment derived from a mammalian Tsg101 protein, wherein the peptide or fragment is effective in reducing HIV particle production, said method comprising:

30

(a) introducing an expression construct into a mammalian cell or cells, wherein the expression construct comprises a portion of the coding sequence for a mammalian *tsg101* gene;

35

(b) introducing HIV into the mammalian cell or cells;

(c) incubating the transfected and infected mammalian cell or cells in a suitable media for a sufficient time and at a temperature of about 37C to obtain a mammalian cell culture comprising mammalian cells which express a gene product encoded by the portion of a coding sequence for a mammalian *tsg101* gene and which produce HIV particles;

(d) measuring the level of particle associated p24 in the mammalian cell culture; and

(e) correlating a reduced level of particle-associated p24, when compared to a control mammalian cell culture which has not been transfected with the expression construct comprising a portion of the coding sequence for a mammalian *tsg101* gene, with the identification of a peptide or fragment effective in reducing HIV particle production.

3. A method for identifying a peptide or fragment derived from a mammalian Tsg101 protein, wherein the peptide or fragment is effective in reducing HIV particle production, said method comprising:

(a) introducing an expression construct into a mammalian cell or cells, wherein the expression construct comprises a portion of the coding sequence for a mammalian *tsg101* gene;

(b) introducing HIV into the mammalian cell or cells;

(c) incubating the transfected mammalian cell or cells in a suitable media for a sufficient time and at a temperature of about 37C to obtain a mammalian cell culture comprising mammalian cells which express a gene product encoded by the portion of a coding sequence for a mammalian *tsg101* gene and which produce HIV particles.

(d) quantifying the HIV particles released from the cells; and

(e) correlating a reduction in HIV particle production, when compared to a control mammalian cell culture which has not been transfected with the expression construct comprising a portion of the coding sequence for a mammalian *tsg101* gene, with the identification of a peptide or fragment effective in reducing HIV particle production.

4. A method for identifying a peptide or fragment derived from a mammalian Tsg101 protein, wherein the peptide or fragment is effective in reducing HIV particle production, said method comprising:

- 5 (a) introducing one or more expression constructs into the mammalian cell or cells, wherein the one or more expression constructs comprises the HIV *gag*, *pol*, and *rev* coding sequences;
- 10 (b) introducing into the mammalian cell or cells; a peptide or fragment derived from a mammalian Tsg101 protein;
- (c) incubating the mammalian cell or cells transfected with the one or more expression constructs and permeated with a peptide or fragment derived from a mammalian Tsg101 peptide, in a suitable media for a sufficient time and at a temperature of about 37C to
15 obtain a cell culture;
- (d) measuring the level of particle associated p24 in the mammalian cell culture; and
- 20 (e) correlating a reduced level of particle-associated p24, when compared to a control mammalian cell culture which has not been permeated with a peptide or fragment derived from a mammalian Tsg101 protein, with the identification of a peptide or fragment effective in reducing HIV particle production.

5. A method for identifying a peptide or fragment derived from a mammalian Tsg101 protein, wherein the peptide or fragment is effective in reducing HIV particle production, said method comprising:

- (a) introducing HIV into a mammalian cell or cells;
- 30 (b) introducing into the mammalian cell or cells; a peptide or fragment derived from a mammalian Tsg101 protein;
- (c) incubating the mammalian cell or cells transfected with HIV and permeated with a peptide or fragment derived from a mammalian Tsg101 protein in a suitable media for a
35 sufficient time and at a temperature of about 37C to obtain a cell culture;

(d) measuring the level of particle associated p24 in the cell culture; and

5 (e) correlating a reduced level of particle-associated p24, when compared to a control mammalian cell culture which has not been permeated with a peptide or fragment derived from a mammalian Tsg101 protein, with the identification of a peptide or fragment effective in reducing HIV particle production.

6. A method for identifying a peptide or fragment derived from a mammalian Tsg101 protein, wherein the peptide or fragment is effective in reducing HIV particle
10 production, said method comprising:

(a) introducing HIV into a mammalian cell or cells;

15 (b) introducing into the mammalian cell or cells, a peptide or fragment derived from a mammalian Tsg101 protein;

(c) incubating the mammalian cell or cells transfected with HIV and permeated with a peptide or fragment derived from a mammalian Tsg101 protein in a suitable media and for a sufficient time, at a temperature of about 37C;

20

(d) quantifying the HIV particles released from the cells; and

(e) correlating a reduction in HIV particle production, when compared to a control mammalian cell culture which has not been permeated with a peptide or fragment
25 derived from a mammalian Tsg101 protein, with the identification of a peptide or fragment effective in reducing HIV particle production.

7. The method of any one of claims 1, 2 or 3 wherein the coding sequence for a mammalian *tsg101* gene is from the UEV A domain of *Tsg101*.

30

8. A peptide or fragment useful for reducing HIV particle production, said peptide comprising at least four contiguous amino acids of a mammalian Tsg101 protein and identified by the method of any one of claims 1-6.

35

9. The peptide or fragment of claim 8 wherein the mammalian Tsg101 protein is a mouse Tsg101 protein (SEQ ID NO:1), human Tsg101 protein (SEQ ID NO:2) or other mammalian Tsg101 protein.

5 10. The peptide or fragment of claim 8 wherein the four contiguous amino acids are from the UEV A domain of a mammalian Tsg101 protein.

11. The peptide of claim 8 wherein the peptide reduces HIV particle production by at least about three-fold.

10

12. A pharmaceutical composition useful for reducing HIV particle production, said composition comprising one or more pharmaceutically acceptable carriers or diluents and a mammalian Tsg101 protein or a fragment thereof.

15 13. A pharmaceutical composition useful for reducing HIV particle production, which comprises one or more pharmaceutically acceptable carriers or diluents and a peptide according to claim 8.

14. The pharmaceutical composition of claim 13 wherein the Tsg101 protein is a
20 mouse Tsg101 protein (SEQ ID NO:1).

15. The pharmaceutical composition of claim 13 wherein the Tsg101 protein is a human Tsg101 protein (SEQ ID NO:2).

25 16. The pharmaceutical composition of claim 13 wherein the four contiguous amino acids are from the UEV A domain of a mammalian Tsg101 protein.

17. A method for identifying a peptide comprising the HIV-1 Gagp6 late (L) domain, PTAPP (SEQ ID NO:3), wherein the peptide inhibits interaction between a Tsg101
30 protein and HIV Gag, said method comprising:

(a) immobilizing on at least a first and second solid support or surface, a peptide comprising the HIV-1 Gag or HIV-1 Gagp6 late (L) domain, wherein the immobilized peptide is linked to a specific anti-Gag or L domain antibody or other linker;

35

(b) incubating at about 4C for a sufficient time, a first reaction mixture comprising labeled Tsg101 protein, a suitable buffer, and a peptide which comprises at least four contiguous amino acids of the HIV-1 Gagp6 late (L) domain;

5 (c) incubating at about 4C for a sufficient time, a second reaction mixture comprising labeled *tsg101* and a suitable buffer;

(d) adding the first reaction mixture of step (b) to the first solid support or surface mixture of step (a) and incubating in a liquid phase at a temperature of about 4C for a
10 sufficient time;

(e) adding the second reaction mixture of step (c) to the second solid support or surface mixture of step (a) and incubating in a liquid phase at a temperature of about 4C for a sufficient time;

15 (f) separating the first solid support or surface from the liquid phase of step (d) to obtain a first solid phase;

(g) separating the second solid support or surface from the liquid phase of step (e) to
20 obtain a second solid phase;

(h) determining the amount of labeled *tsg101* in the first solid phase of step (f) and in the second solid phase of step (g); and

25 (i) correlating a decrease in the amount of labeled *tsg101* in the first solid phase of step (f) when compared to the amount of labeled *tsg101* in the second solid phase of step (g) with the identification of a peptide comprising at least four contiguous amino acids of the HIV-1 Gagp6 late (L) domain wherein said peptide inhibits interaction
30 between a Tsg101 protein and HIV Gag.

18. The method of claim 17 wherein the Tsg101 protein is a mouse, human, or other mammalian Tsg101 protein.

35

19. A peptide useful for inhibiting interaction between Tsg101 protein and HIV Gag, said peptide comprising at least four contiguous amino acids of the HIV-1 Gagp6 late (L) domain, PTAPP (SEQ ID NO:3) and identified by the method of claim 17.

5 20. The peptide of claim 19 further comprising at least five contiguous amino acids of ALQSRPEPTAPPEES (SEQ ID NO:4).

21. The peptide of claim 19 or 20 wherein the peptide causes at least about a three-fold reduction in TSG101-HIV Gag interaction.

10

22. A pharmaceutical composition useful for inhibiting interaction between TSG101 and HIV Gag, said composition comprising one or more pharmaceutically acceptable carriers or diluents and a peptide according to claim 19 or 20.

15 23. A method of treating a patient infected with HIV or other retrovirus which comprises administering a therapeutically effective amount of a peptide according to claim 8.

24. A method of treating a patient infected with HIV or other retrovirus which comprises administering a therapeutically effective amount of a peptide according to claim 9.

20

25. A method of treating a patient infected with HIV or other retrovirus which comprises administering a therapeutically effective amount of a peptide according to claim 10.

25 26. A method of treating a patient infected with HIV or other retrovirus which comprises administering a therapeutically effective amount of a peptide according to claim 11.

27. A method of treating a patient infected with HIV or other retrovirus which
30 comprises administering a therapeutically effective amount of a peptide according to claim 19 or 20.

28. A method of treating a patient infected with HIV or other retrovirus which
comprises administering a therapeutically effective amount of a pharmaceutical composition
35 according to claim 12.

29. A method of treating a patient infected with HIV or other retrovirus which comprises administering a therapeutically effective amount of a pharmaceutical composition according to claim 13.

5 30. A method of treating a patient infected with HIV or other retrovirus which comprises administering a therapeutically effective amount of a pharmaceutical composition according to claim 14.

31. A method of treating a patient infected with HIV or other retrovirus which
10 comprises administering a therapeutically effective amount of a pharmaceutical composition according to claim 15.

32. A method of treating a patient infected with HIV or other retrovirus which
15 comprises administering a therapeutically effective amount of a pharmaceutical composition according to claim 16.

33. A method of treating a patient infected with HIV or other retrovirus which
comprises administering a therapeutically effective amount of a pharmaceutical composition
20 according to claim 22.

34. A method for identifying a peptide or fragment derived from a mammalian
Tsg101 protein, wherein said peptide or fragment is effective in reducing HIV particle
production, said method comprising:

25 (a) measuring a level of HIV particles released in a culture of mammalian cells,
wherein said mammalian cells comprise an expression construct comprising a portion of the
coding sequence of a mammalian *tsg101* gene such that said mammalian cells express a gene
product encoded by said portion of said coding sequence of said mammalian *tsg101* gene and
are infected by HIV virus; and

30 (b) comparing said level of HIV particles to a level of HIV particles released in a
culture of control mammalian cells under similar conditions, wherein said control
mammalian cells do not comprise an expression construct comprising a portion of a coding
sequence for a mammalian *tsg101* gene and are infected by HIV virus,

35

wherein said level of HIV particles measured in step (a) compared to said level of HIV particles of said culture of control mammalian cells below a predetermined threshold level identify said gene product encoded by said portion of said coding sequence of said mammalian *tsg101* gene as effective in reducing HIV particle production.

5

35. A method for identifying a peptide or fragment derived from a mammalian Tsg101 protein, wherein said peptide or fragment is effective in reducing HIV particle production, said method comprising:

10 (a) measuring a level of HIV particles released in a culture of mammalian cells, wherein said mammalian cells comprise (i) an expression construct comprising a portion of the coding sequence of a mammalian *tsg101* gene and (ii) one or more expression constructs comprising the HIV *gag*, *pol*, and *rev* coding sequences such that said mammalian cells express a gene product encoded by said portion of said coding sequence of said mammalian
15 *tsg101* gene and gene products encoded by said HIV *gag*, *pol* and *rev* genes; and

(b) comparing said level of HIV particles released to a level of HIV particles released in a culture of control mammalian cells under similar conditions, wherein said control mammalian cells comprise one or more expression constructs comprising the HIV *gag*, *pol*,
20 and *rev* coding sequences such that said mammalian cells express gene products encoded by said HIV *gag*, *pol* and *rev* genes, and wherein said control mammalian cells do not comprise an expression construct comprising a portion of a coding sequence for a mammalian *tsg101* gene,

25 wherein said level of HIV particles measured in step (a) compared to said level of HIV particles released in said culture of control mammalian cells below a predetermined threshold level identify said gene product encoded by said portion of said coding sequence of said mammalian *tsg101* gene as effective in reducing HIV particle production.

30 36. A method for identifying a peptide or fragment derived from a mammalian Tsg101 protein, wherein said peptide or fragment is effective in reducing HIV particle production, said method comprising:

(a) measuring a level of HIV viral particle released in a culture of mammalian cells,
35 wherein said mammalian cells comprise (i) an expression construct comprising a portion of

the coding sequence of a mammalian *tsg101* gene and (ii) an expression construct comprising the HIV *gag* coding sequence such that said mammalian cells express a gene product encoded by said portion of said coding sequence of said mammalian *tsg101* gene and a gene product encoded by said HIV *gag* gene; and

5

(b) comparing said level of HIV viral particle released to a level of HIV viral particle released in a culture of control mammalian cells under similar conditions, wherein said control mammalian cells comprise one or more expression constructs comprising said HIV *gag* coding sequence such that said mammalian cells express a gene product encoded by said HIV *gag* gene, and wherein said control mammalian cells do not comprise an expression
10 construct comprising a portion of a coding sequence for a mammalian *tsg101* gene,

wherein said level of HIV viral particle measured in step (a) compared to said level of HIV viral particle released in said culture of control mammalian cells below a predetermined
15 threshold level identify said gene product encoded by said portion of said coding sequence of said mammalian *tsg101* gene as effective in reducing HIV particle production.

37. The method of claim 34, 35, or 36, wherein said level of HIV particles released in a culture of mammalian cells and said level of HIV particles released in a culture of control
20 mammalian cells are represented by measured levels of particle associated p24.

38. The method of claim 34, 35, or 36, wherein said portion of said coding sequence of said mammalian *tsg101* gene is a sequence in the UEV A domain of said *Tsg101* gene.

25 39. The method of claim 38, wherein said predetermined threshold level is a two-fold reduction of said level of HIV particles measured in step (a) compared to said level of HIV particles of said culture of control mammalian cells.

40. The method of claim 38, wherein said predetermined threshold level is a four-fold
30 reduction of said level of HIV particles measured in step (a) compared to said level of HIV particles of said culture of control mammalian cells.

41. The method of claim 40, wherein said predetermined threshold level is a 90%
35 reduction of said level of HIV particles measured in step (a) compared to said level of HIV particles of said culture of control mammalian cells.

42. The method of claim 41, wherein said predetermined threshold level is a 95% reduction of said level of HIV particles measured in step (a) compared to said level of HIV particles of said culture of control mammalian cells.

5 43. The method of claim 42, wherein said predetermined threshold level is a 99% reduction of said level of HIV particles measured in step (a) compared to said level of HIV particles of said culture of control mammalian cells.

10 44. The method of claim 43, wherein said predetermined threshold level is a 99.5% reduction of said level of HIV particles measured in step (a) compared to said level of HIV particles of said culture of control mammalian cells.

15 45. A method for identifying a peptide or fragment derived from a mammalian Tsg101 protein, wherein said peptide or fragment is effective in reducing retrovirus production, said method comprising identifying a peptide that binds to a peptide comprising the PTAP motif of a retroviral protein.

46. The method of claim 45, wherein said retrovirus is a lentivirus.

20 47. The method of claim 46, wherein said lentivirus is HIV-1 virus.

48. The method of claim 46, wherein said lentivirus is HIV-2 virus.

25 49. The method of claim 46, wherein said lentivirus is simian immunodeficiency virus.

50. The method of claim 45, wherein said peptide comprising the PTAP motif is a HIV Gag protein.

30 51. The method of claim 45, wherein said peptide comprising the PTAP motif is the peptide of SEQ ID NO:4.

35 52. A method for identifying a peptide comprising a PTAP motif, wherein said peptide is effective in reducing retrovirus production, said method comprising identifying a peptide comprising a PTAP motif which binds to a TSG101 protein or a fragment thereof.

53. The method of claim 52, wherein said retrovirus is a lentivirus.

54. The method of claim 53, wherein said lentivirus is HIV-1 virus.

5 55. The method of claim 53, wherein said lentivirus is HIV-2 virus.

56. The method of claim 53, wherein said lentivirus is simian immunodeficiency
virus.

10 57. The method of claim 52, wherein said peptide comprises the sequence of SEQ ID
NO:4.

58. The method of any one of claims 34-36 and 45-57, wherein said tsg101 gene is a
human tsg101 gene.
15

20

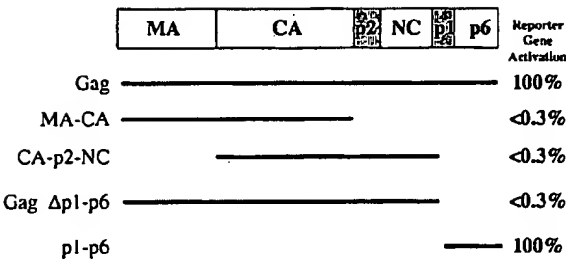
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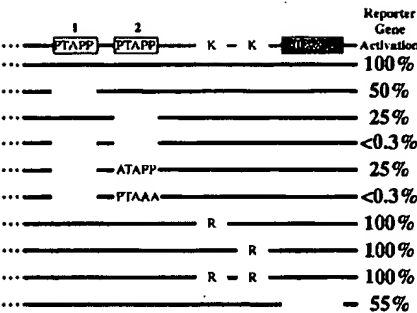
35

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A. Pr55^{Gag}

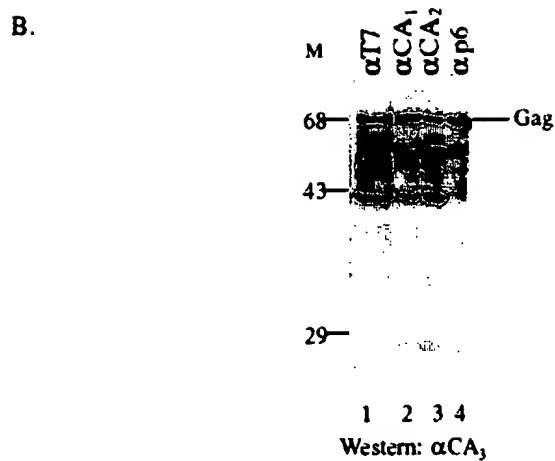
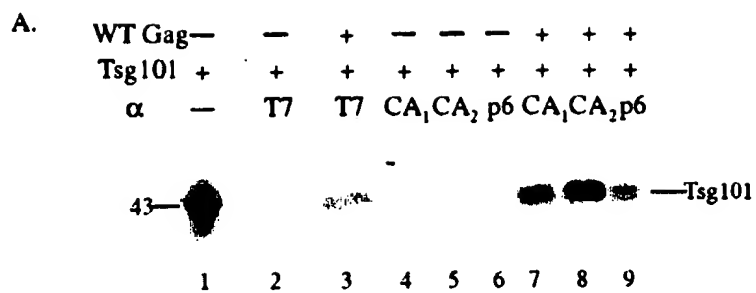


B. p1-p6



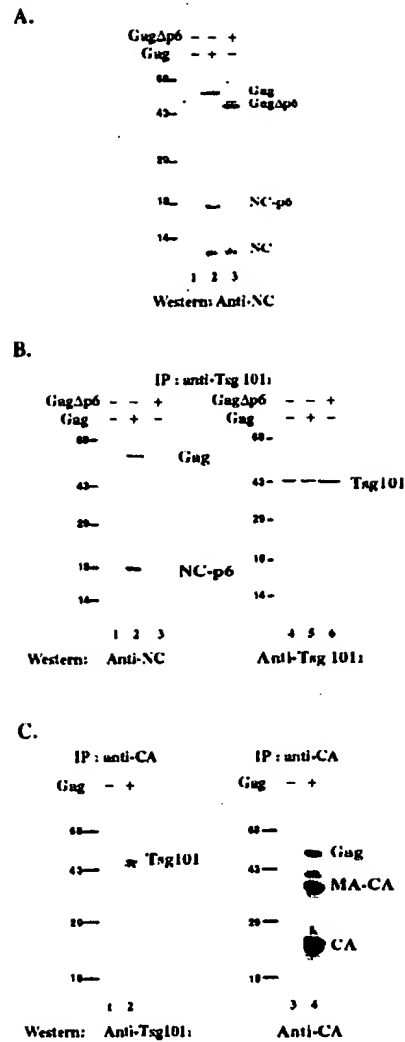
FIGS. 1A-1B

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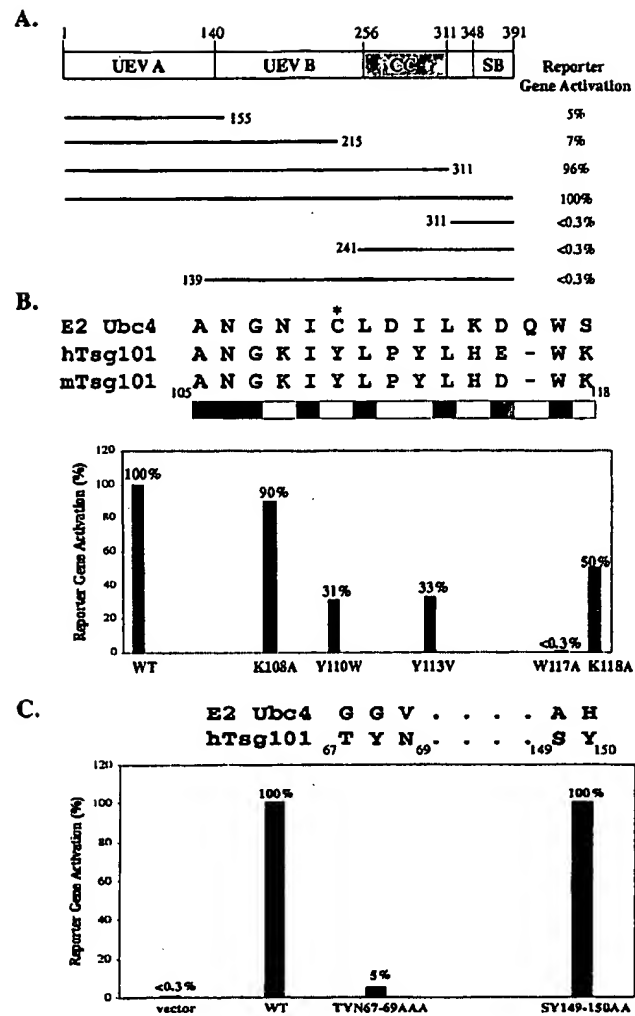
FIGS. 2A-2B

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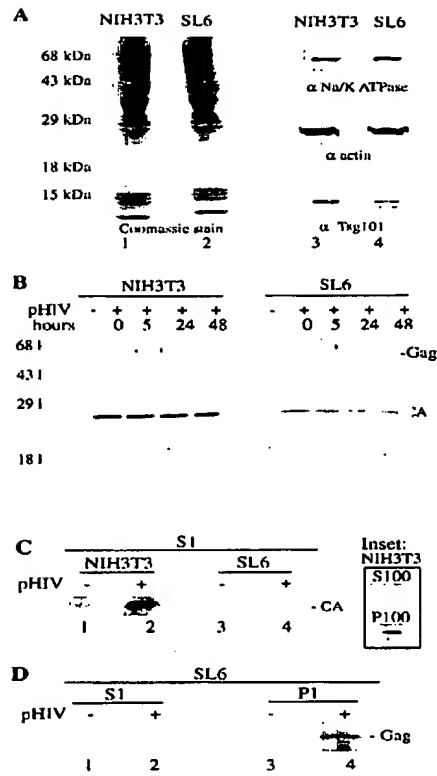
FIGS. 3A-3C

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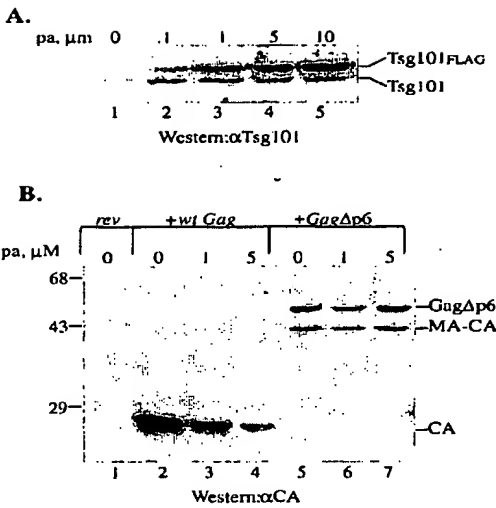
FIGS. 4A-4C

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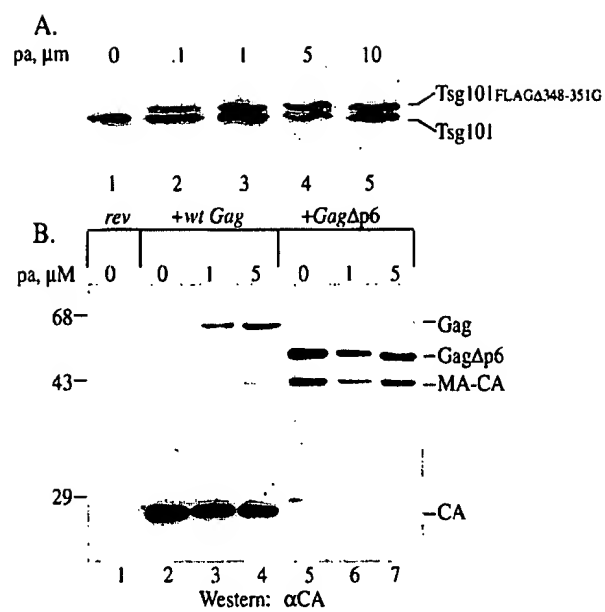
FIGS. 5A-5D

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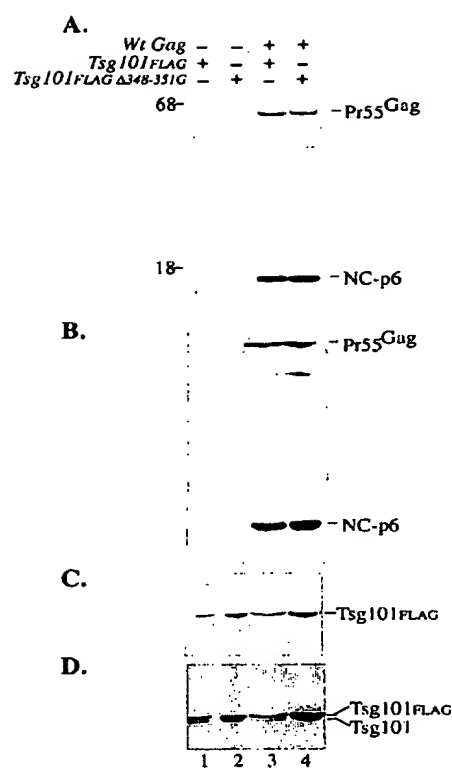
FIGS. 6A-6B

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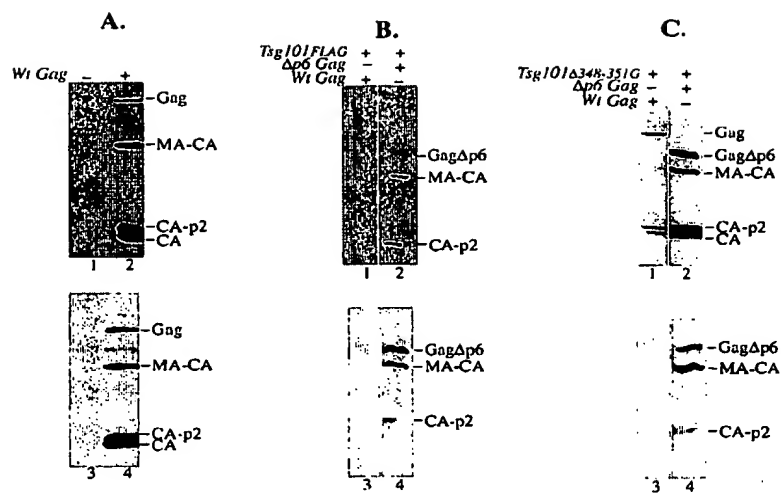
FIGS. 7A-7B

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FIGS. 8A-8D

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FIGS. 9A-9C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/15965

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : 424/208.1; 455/6, 7.1, 91.4, 320.1, 325, 455

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/208.1; 455/6, 7.1, 91.4, 320.1, 325, 455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,892,016 A (BRIE et al) 06 April 1999, please see entire document.	1-58
A, P	US 6,248,523 B1, (COHEN et al) 19 June 2001, please see entire document.	1-58



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"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 JUNE 2002

Date of mailing of the international search report

20 AUG 2002

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Facsimile No. (703) 305-3230

Authorized officer

LAURIE SCHEINER

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/15965

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

A61K 39/21; A01N 43/04; G01N 33/53; C12Q 1/68; C12N 15/00, 15/64, 5/00, 15/63

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